Conservation of the platypus (*Ornithorhynchus anatinus*): Development of a framework to assess the health of wild platypus populations.

James W Macgregor BA VetMB MVS

This thesis is presented for the degree of Doctor of Philosophy of Murdoch University 2015.

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

James W Macgregor BA VetMB MVS

Abstract

A wide range of factors, including individual animal health, genetic diversity and demographics, are associated with wildlife population declines and investigation of these factors may be more sensitive in detecting early impacts on wildlife populations, than estimates of population size alone. Defining wildlife population health as the ability of a wildlife population to remain viable in the long term, this project developed and implemented a holistic health assessment framework for platypuses to gather baseline data, to investigate environmental, temporal and individual patterns within this data, and to provide insights into potential threatening processes.

Platypus distribution and population density in two river catchments in northwest Tasmania were investigated in a live capture/release field study during which 154 individuals were captured. The effect on capture numbers of broad habitat characteristics was investigated. A survey of public sightings provided additional information on platypus distribution and population density. The novel use of in-stream microchip readers to monitor platypus movements/survivorship was developed. Data was collected on the timing and frequency of platypus movements, as well as continued use of monitoring sites by individuals captured in this study and in a study three to six years earlier. The timing of the breeding season in Tasmania was investigated using hormonal, ultrasonographic and remote monitoring observations. Genetic diversity and geographical distribution of alleles at the Major Histocompatibility Complex Class II DZB locus was also investigated. The reliability of existing and novel body condition indices was studied. The prevalence of exposure to a range of parasitic, fungal and bacterial agents was determined. Haematology and biochemistry reference intervals were produced.

Little evidence was found that the two study populations were in poor health. Baseline population health data, that for many species has been absent when population declines have occurred, was collected for platypuses; and the project's general approach will serve as a template for similar research in other species.

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Photo: Helen Robertson

Publications

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Glossary of terms and abbreviations

Adult female platypus	Older than ~9 months; no spur or spur sheath present †
Adult male platypus	Male platypus older than ~15 months; spur but no spur sheath present †
A/G ratio	Albumin : globulin ratio
ANOVA	Analysis of variance
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
Bands	Band neutrophils
BCI 1	Body condition index 1; body mass/ total body length^{3}
BCI 2	Body condition index 2; body mass/ bill width$^{3.2}$
°C	Degrees Celcius
CK	Creatinine Kinase
DOY	Day Of Year
DOY sine wave parameter	Parameter for each DOY that varies sinusoidally over the course of a year
Fieldwork site	Fyke net site
GGT	Gamma-glutamyl transferase
GLDH	Glutamate dehydrogenase
GPS	Global Positioning System
Hb	Haemoglobin

Inglis Catchment	Area of northwest Tasmania mostly drained by the Inglis River, but also containing smaller river basins
Inglis River Catchment	Area of Inglis Catchment that is drained by the Inglis River at Wynyard
Juvenile female platypus	<12 months; no spur but spur sheath retained March - December †
Juvenile male platypus	<12 months of age; Spur and full spur sheath present January - June, spur and full or partial spur sheath present July - December †
K	Potassium
LM	Linear measure of body size
Local forest area	Forest area within a 500m radius of a fieldwork site
MCH	Mean Corpuscular Haemoglobin
MCHC	Mean Corpuscular Haemoglobin Concentration
MCV	Mean Corpuscular Volume
MHC	Major Histocompatibility Complex
MHC class II DZB	Protein involved with presenting pathogenic peptides to cytotoxic T cells.
Mid-tail fat depth	Dorso-ventral thickness of fat adjacent to the bone and musculature of tail at the mid-point along the tail's length
Mid-tail fat area	Cross-sectional area of the fat in the tail at the mid-point along the tail's length

Monitoring site	Location of in-stream monitoring
Mucormycosis	Disease of platypuses, caused by the fungal organism *Mucor amphibiorum*, that can lead to cutaneous ulcers/nodules, lesions in internal organs, and death.
Na	Sodium
Nasopharyngeal response	Sudden onset apnoea and bradycardia occurring on exposure of the nasal cavities to irritant chemicals including isoflurane
Net pair hour	Measure of capture effort defined as a pair of nets in place at a site for one hour
OLS	Ordinary Least Squares linear regression
PCV	Packed Cell Volume
PCR	Polymerase chain reaction
Phomopsis/Diaporthe	A fungal genus
Platypus observation	Any single or multiple recording of a microchip by an instream antenna, at least 30 minutes before and after other observations of the same microchip.
r^2	Coefficient of determination
RCC	Red Cell Count
Reference curve	Reference interval that varies sinusoidally over the course of a year
Relative tail volume	Tail volume measured by water displacement/tail length

Resident individuals	Platypuses captured or observed at a site that is part of a consistent home range
Retirement	Period of ~20 days spent by females in nesting burrows for egg laying and incubation.
RFD 1	Relative Fat Depth 1; 10^6*mid-tail fat depth$^{1.7}$/total body length^3
RFD 2	Relative Fat Depth; 10^6*mid-tail fat depth$^{1.7}$/bill width$^{3.2}$
RMA	Reduce Major Axis regression
RTFV 1	Relative Tail Fat Volume 1; 10^4*tail fat volume/total body length^3
RTFV 2	Relative Tail Fat Volume 2; 10^4*tail fat volume/bill width$^{3.2}$
Seabrook Creek Catchment	Area of Inglis Catchment that is drained by Seabrook Creek
SD	Standard deviation
Subadult female platypus	12-14 months; no spur, spur sheath retained January - February †
Subadult male platypus	12-18 months of age; spur and partial spur sheath present January - June †
Subcatchment	Subdivision of a river catchment based on geographic, topographic and hydrological features
Subcatchment forest area	Proportion of subcatchment that has forest cover
Transient individuals	Platypuses captured or observed at a site that is not part of a consistent home range

Trovan®ANT 612	~500mm square pass-over microchip reading antenna
Trovan®ANT C600	600mm diameter pass-through microchip reading antenna
tbl	Total body length
TFI	Cross-sectional area of the tail at its mid-point (determined by measuring height and width and assuming the area has the shape of an arc of a circle) divided by tail length
T protein	Total Protein
TVI	Tail Volume Index – determined by assessing the degree to which the lateral edges of the tail can be folded downwards with gentle downward
WCC	White Cell Count

†Note that because of variable times of spur sheath loss, there is overlap between the age categories, as described in Sections 2.2.5 and 7.2.2.

Chapter 1.

Introduction

1.1 PLATYPUS BIOLOGY

The platypus (*Ornithorhynchus anatinus*) is a semi-aquatic mammal found only in Eastern Australia, with its distribution ranging from Cooktown in Queensland to Tasmania. It is one of only five extant species of the Order Monotremata (Sub-class Prototheria), the only egg-laying mammals, and the only extant species of the family Ornithorhynchidae (Flannery and Groves, 1998; Booth, 2003). Platypuses are sexually dimorphic in terms of weight – the males being approximately 50% heavier than the females at any particular location (Grant and Temple-Smith, 1983; Connolly and Obendorf, 1998; Gust and Griffiths, 2011). Mean values for male and female body mass have been in the ranges of 1.2-1.8 kg and 0.8-1.2 kg, respectively, in mainland platypuses (Grant and Temple-Smith, 1983; Gardner and Serena, 1995) and 1.5-2.5 kg and 0.9-1.7 kg, respectively, in Tasmanian platypuses (Connolly and Obendorf, 1998; Bethge, 2002; Koch *et al.*, 2006; Macgregor, 2008; Gust and Griffiths, 2011).

Platypus are solitary animals except during the breeding season, when males seek out females but females continue to avoid males except for a receptive period of 4-6 days (Hawkins and Battaglia, 2009). A female platypus can lay up to three eggs, each approximately 14 x 14 x 17 mm in size, after a gestation period of 15-21 days (Hill and Gatenby, 1926; Hawkins and Battaglia, 2009). It is believed she incubates the egg(s) on her ventral abdomen in a curled position for approximately 10-12 days (Grant and Dawson, 1978b; Hawkins and Battaglia, 2009). After hatching, the young platypuses feed on milk from their mothers (Grant *et al*, 1983). This milk is produced in two mammary glands and secreted via a network of ducts leading to secretory patches similar in structure to a nipple in other mammals except that they do not protrude (Grant *et al.*, 1983; Grant, 2007). The juvenile platypuses remain in the nesting burrow while

the mother spends increasing amounts of time in the water feeding (Grant, 2007; Hawkins and Battaglia, 2009). At approximately four months of age, the young emerge from the burrow and become independent within days (Grant, 2007; Hawkins and Battaglia, 2009). Reproductive endocrinology and evidence of lactation has demonstrated that the breeding season occurs during August-September in mainland platypuses (Grant *et al.*, 1983; Jakubowski *et al.*, 1998; New *et al.*, 1998). Progesterone assay results in female platypuses have shown evidence that the onset of the breeding season occurs later with increasing latitude in mainland platypuses (Jakubowski *et al.*, 1998). Similarly, observations that capture of newly-emerged juveniles in Tasmanian field studies lags behind that on the mainland by two to three months suggest that the breeding season occurs even later in Tasmania (Grant *et al.*, 1983; Grant and Temple-Smith, 1983; Connolly and Obendorf, 1998; Munks *et al.*, 1998; Munks *et al.*, 2000; Grant, 2004).

The monotreme fossil record starts with a specimen from 115 million years ago (Rich *et al.*, 2001; Musser, 2003). The ornithorhynchid fossil record includes four extinct species and dates back to a 62 million year old specimen found in Patagonia, Argentina (Pascual *et al.*, 1992; Pian *et al.*, 2013). Although this specimen implies that ornithorynchids were once distributed across the ancient supercontinent of Gondwana, all other specimens from the family Ornithorhynchidae have been found in Australia (Musser, 2003). These include the recently reported *Obdurodon tharalkooschild*, which is estimated to have been over 70 cm in length and was probably not a direct ancestor of the modern platypus (Pian *et al.*, 2013). The earliest specimens of *Ornithorhynchus anatinus* date from approximately 100,000 years ago (Musser, 1998).

This fossil record and genomic evidence suggest that monotremes diverged from the rest of the mammals 160-200 million years ago (Musser, 2003; Bininda-Emonds *et al.*, 2007). While certain aspects of platypus physiology (including reproduction - the laying of eggs in particular, venom production and electroreception) appear superficially to suggest it remains a primitive species, closer examination implies that the platypus is an example of mosaic evolution in which "archaic features occur alongside highly specialised or advanced features in the same animal or plant" (Musser, 2003, p.928). In relation to reproduction, some genes encoding proteins relating to egg and sperm production have been retained from before divergence from the rest of the mammals and others from ancestors that platypuses have in common with reptiles (Warren *et al.*, 2008). The genes for the milk protein casein appear to have been retained in platypuses from before divergence from eutherian mammals (Warren *et al.*, 2008) and there is at least one similarity in the sex chromosomes to those of birds (Grützner *et al.*, 2004; El-Mogharbel *et al.*, 2007). However, electoreception and venom production, both characteristics that appear archaic (the former most commonly seen in fish and amphibians, the latter most commonly seen in reptiles, fish and amphibians), have actually evolved independently in the platypus, as described below.

Electroreception is an important part of prey location as platypuses forage for invertebrates on the floor of streams or lakes. Although the exact mechanism has yet to be determined it appears that prey location by platypuses involves the use of two types of receptors on the bill (Schleich, 1986; Grant, 2007). One of these types of receptor is a group of push rod organs which are present in large numbers in the skin of the bill. These are unique to monotremes but similar to the Eimer Organ of moles (Gregory *et*

al., 1988). They occur in three types in the platypus and are assumed to be mechanoreceptors (Gregory *et al.*, 1988).

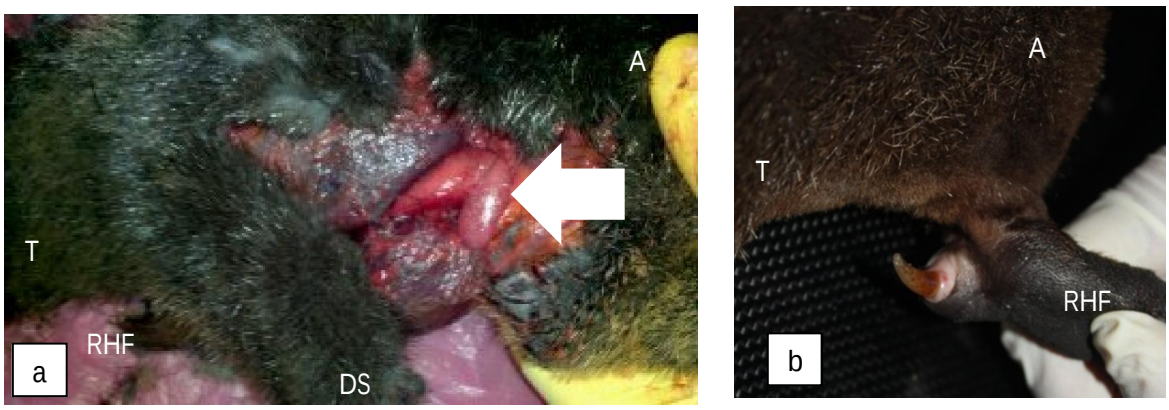
Figure 1.1. The bill of the platypus (*Ornithorhynchus anatinus*), containing electroreceptors **Photo: Christina Shaw.**

In greater numbers in the skin of the platypus bill are receptors located in the duct lining of a mucus secreting gland (Schleich, 1986; Gregory et al., 1988), which have been shown to be sensitive to DC electrical fields and are thought likely to be used to detect electrical activity in the bodies of their prey (Schleich, 1986; Gregory *et al.*, 1988). Electroreception is a sense that, other than in monotremes, has only been found in certain species of fish and amphibians and a single dolphin species, the Guiana dolphin (*Sotalia guianensis*) (Schleich, 1986; Czech-Damal *et al.*, 2011). Location of these electroreceptors in gland ducts is thought to be an adaptation to the semi-aquatic lifestyle of the platypus, allowing conductivity to be maintained and avoiding damage due to desiccation when the animal leaves the water (Schleich, 1986). The receptors are connected to the cerebral cortex via the trigeminal nerve (Schleich, 1986; Gregory *et al.*,

1988). While the receptors of the platypus are similar to those found in fish, their structural design is unique and their innervation by the trigeminal nerve, along with those of the echidna, is not found in any other Order of animal (Schleich, 1986; Gregory *et al.*, 1988). These facts suggest that platypus electroreceptors have evolved independently from those in fish and amphibians and are highly specialised for their environment (Schleich, 1986; Gregory *et al.*, 1988).

Venom production is very uncommon amongst mammals (Warren *et al.*, 2008); usually occurring in reptiles, fish and amphibians. The male platypus has a venom-producing gland (crural gland) located in the proximal part of each hindlimb (Figure 1.2a). Each crural gland is connected to a keratinous spur, adjacent to the os calcaris in the hock (Figure 1.2b), through which the venom is delivered, a system unique amongst mammals (Warren *et al.*, 2008).

Figure 1.2. a) Platypus crural gland (arrow) at necropsy, and b) Spur on hindlimb RHF= right hind foot, T=tail, A=abdomen, DS=dissected skin **Photo b: Helen Robertson.**



The venom produced by the crural gland contains 19 different fractions including defensin-like peptides, C-type naturiuretic peptides, nerve growth factors, and uncharacterised protein and non-protein components (Whittington *et al.*, 2008). The

effects of these components have not been fully characterised, but nerve growth factors are also found in snake venoms and C-type naturiuretic peptides have been found in the venom of the South American pit viper (Whittington *et al.*, 2008). The defensin-like peptides have similar structures to crotamine-like peptides present in the venom of some sauropsid reptiles (Whittington *et al.*, 2008). Genomic investigation has shown that while they have evolved from similar antimicrobial peptides to those of sauropsids, these venom molecules have evolved independently in platypuses (Whittington *et al.*, 2008).

1.2 SEMI-AQUATIC LIFESTYLE

The platypus is found associated with water bodies with varied habitat characteristics and spends its time either feeding in water or resting in a burrow (Grant and Bishop, 1998). There are two types of platypus burrows: camping burrows and nesting burrows (Grant and Bishop, 1998).

Camping burrows are usually quite short (Grant and Bishop, 1998) and used by platypuses on a daily basis as rest sites. They are frequently located in the earth banks of water bodies but have also been found in river debris, surrounding vegetation and caves (Serena, 1994; Woon, 1995; Bishop, 1998, Otley et al., 2000; Munks et al.,2004). The entrance to a camping burrow in a river bank may be submerged or above the water level (Serena, 1994). All platypuses observed in camping burrows by Serena (1994) rested within 3 m (and usually between 1 and 2 m) of the entrance. Individual platypuses have been observed to use multiple burrows in their home range over periods of days or weeks (Grant, 1983; Serena, 1994; Gust and Handasyde, 1995; Serena *et al.*, 1998; Otley *et al.*, 2000). Such burrows have been observed to be used by different

platypuses at different times, as well as by pairs of platypuses at the same time (Serena, 1994; Gust and Handasyde, 1995; Otley *et al.*, 2000).

Nesting burrows, where females rear their young, are generally longer and more complex than camping burrows and are almost always located in earth banks (Grant and Bishop, 1998; Grant, 2007). They can be up to 30 m long with several chambers, of which one is lined with fur and other nesting material (Grant, 2007). Serena (1994) observed that females in nesting burrows rested on average 5 m from the burrow entrance.

Platypuses generally enter and exit a burrow once a day (Serena, 1994). Different studies have recorded that platypuses are out of their burrow and active for 7.3-12.4 h (Serena (1994), 10 h (Gust and Handasyde, 1995) and 8.5-16 h (Otley *et al.* (2000) per day. Bethge (2002) observed a mean duration of activity periods of 12.5 h with a range of 2.77 to 29.83 h, and found activity periods to be longer between August and November. Serena (1994) and Gust and Handasyde (1995) found platypuses to be active mostly at night, although they did record diurnal activity. Otley *et al.* (2000) and Bethge (2002) observed a higher proportion of platypuses to be diurnally active at Lake Lea in Tasmania, particular for females in winter. This may be a result of increased costs of thermoregulation in Tasmania, particularly in winter, reduced predation pressure in the absence of foxes, or other ecological factors (Otley *et al.*, 2000; Temple-Smith, 2000).

Platypuses feed continuously while they are out of the burrow, even in winter (Otley *et al.*, 2000; Bethge, 2002). A platypus must dive to the bottom of the water body to find and collect its food (Grant, 2007). It then returns to the surface to breathe and masticate

the food. Food is stored in cheek pouches before mastication between grinding pads at the back of the mouth (Faragher *et al.*, 1979). By analysing the contents of the cheek pouches, the diet of the platypus has been found to consist primarily of benthic macroinvertebrates (Grant and Carrick, 1978; Faragher *et al.*, 1979; Grant, 1982). Although there is some evidence of a degree of selection amongst these species (McLachlan-Troup, 2007), prey appears largely to be eaten in approximate proportion with their abundance on the floor of the water body (Faragher *et al.*, 1979; Grant, 1982).

During diving there is a large drop in heart rate, which presumably leads to reduced tissue perfusion (Johansen *et al.*, 1966; Evans *et al.*, 1994), which is analogous to the dive response in other species. However, there is little evidence of reliance on anaerobic metabolism during diving due to the high oxygen carrying capacity of blood, the storage of oxygen in the lungs after a large inspiration before diving and the relatively short dive durations (Johansen *et al.*, 1966; Whittington and Grant, 1984; Evans *et al.*, 1994). Kruuk (1993) observed an average dive time of 35 s, with a maximum of 75 s in wild platypuses. For captive platypuses, Evans *et al.* (1994) found that most dives lasted between 30 s and 4 min, but observed a maximum dive time of 11 min. Bethge (2002) observed a mean dive depth of 1.21 m and a maximum dive depth of 8.77 m, with 98.3% of dives recorded at depths of less than 3 m (Bethge, 2002).

1.3 PLATYPUS CONSERVATION STATUS

Although there is some evidence of local population reductions since European settlement of Australia, there appears to have been little change in the overall distribution of the platypus in the last two centuries (Connolly and Obendorf, 1998; Grant and Bishop, 1998; Rohweder and Baverstock, 1999; Grant *et al.*, 2000). As a

result the platypus is listed as ‘least concern’ by the IUCN (IUCN, 2011) and is not listed on Australian State or Commonwealth threatened species schedules, with the exception of South Australia where the platypus appears to be extinct from most of its limited distribution at the time of European settlement and is listed as vulnerable (Grant, 2009). However, because of its dependence on river systems for its survival some authors consider the species vulnerable throughout its distribution (Grant and Llewellyn, 1991; Turnbull, 1998), although the collection of data to demonstrate this is complicated by difficulties in assessing population size/density in this species (Grant, 2012).

Various estimates of population density and size have been made using remote observation and/or live capture and release (Grant and Carrick, 1978; Serena, 1994; Serena and Williams, 1997; Turnbull, 1998; Fox *et al.*, 2004), but these estimates are difficult to verify because of the limitations of the techniques used (Temple-Smith, 2000). Remote observation has the disadvantage that it is impossible to count platypuses since individuals rarely have distinctive external features and monitoring therefore relies on indices rather than absolute platypus numbers (Woon, 1995).

Live capture and release allows individual identification, however there are the following limitations on the use of this method for estimating platypus population size:
1) It is not possible to know if all platypuses in an area have been captured which may produce underestimations of population density (Serena, 1994; Serena and Williams, 1997).

2) The home range of some of the platypuses captured may extend beyond the study area, and others may only transiently occupy an area, leading to overestimations of population density (Seber, 1986; Serena, 1994; Serena and Williams, 1997).

3) Capture rates are low and their use as a measure of platypus population densities complicated by varying suitability of sites for use of nets, varying fieldwork schedules and differing likelihoods that individual platypuses will be captured (Edwards *et al.*, 2000). Different capture rates at different times of day have been reported in platypus research projects (Bethge, 2002; Serena and Williams, 2012).

4) Net avoidance by platypuses after initial capture has been reported (Connolly and Obendorf, 1998; Stewart, 2001; Griffiths *et al.*, 2013). Grant (2004) reported that, during the course of a number of studies in the Shoalhaven River in New South Wales over a 30 year period, 64% of adult male platypuses and 49% of adult female platypuses were only captured once. Connolly and Obendorf (1998) reported a recapture rate of 22% and considered that platypuses became net shy in heavily trapped areas. A low recapture rate in combination with a presumed relatively low population density means that the mark-recapture method is not useful for estimating platypus population numbers.

5) Populations of platypuses are unlikely to be distributed homogenously, making interpretation of live capture data difficult (Seber, 1986).

6) Live platypus capture is labour intensive and time-consuming (Woon, 1995).

1.4 OBSERVED AND POTENTIAL THREATS TO PLATYPUS POPULATIONS

1.4.1 Anthropogenic threats

Various authors have described known causes of death in platypuses (Grant and Llewellyn, 1991; Connolly and Obendorf, 1998; Munday et al., 1998, Serena and Williams, 2010). Grant and Llewellyn (1991) and Grant and Temple-Smith (2003) reviewed threats to platypuses that might result from human activities based on knowledge of the habitat requirements of this species, particularly the need for a source of food and suitable sites for burrows. Table 1.1 lists anthropogenic threats that have been either observed or proposed to have effects on platypuses.

Table 1.1. Observed and potential anthropogenic threats to platypuses.

Threatening process	Subcategories of process	Observed effect on platypuses	Proposed effect on platypuses	Source
Forestry	Increased water run-off from logging operations		Changes in communities of benthic invertebrates in waterbodies as a result of changes in water flow and sedimentation.	Grant and Llewellyn (1991); Grant and Temple-Smith (2003)
	Damage to riparian habitat		Changes in communities of benthic invertebrates. Loss of burrowing sites.	Grant and Temple-Smith (2003)
Agriculture	Alteration of stream flow		Changes in communities of benthic invertebrates in waterbodies due to changes in microenvironments.	Grant *et al.* (1991)
	Stream bank erosion		Loss of suitable sites for burrow construction.	Grant *et al.* (1991)
	Increased water runoff		Increased organic matter, inorganic nutrients and sedimentation leading to changes in benthic invertebrate communities, and pesticides leading to effects on platypuses and their prey.	Grant *et al.* (1991)
Urban development		Reduced numbers of platypus sightings in larger cities	Habitat degradation	Grant and Temple-Smith (2003)
Introduced species	High densities of willows in small streams		Increased flooding, reduced summer flows, loss of habitat for aquatic macroinvertebrates.	Grant and Temple-Smith (2003)
	Dogs	Injury/death		Connolly and Obendorf (1998); Munday *et al.* (1998)
	Foxes	Injury/death		Connolly and Obendorf (1998); Munday *et al.* (1998)

Table 1.1 (Cont'd).

Threatening process	Subcategories of process	Observed effect on platypuses	Proposed effect on platypuses	Source
Fisheries bycatch		Drowning in inappropriately set nets		Grant and Llewellyn (1991); Connolly and Obendorf (1998); Munday *et al.* (1998); Grant (2004)
Dams, weirs and culverts			Barriers to platypus movement. Changes in communities of benthic invertebrates. Reduced access to benthic invertebrates in water >10m in depth.	Grant and Temple-Smith (2003)
Salinity			Effects on osmoregulation and electrolocation of prey. Changes in communities of benthic invertebrates in water body	Grant and Temple-Smith (2003) Grant *et al.* (1991)

1.4.2 Mucormycosis

Platypuses in certain river catchments in Tasmania may suffer from mucormycosis, a disease caused by the fungal organism *Mucor amphibiorum* (Obendorf *et al.*, 1993). The reported clinical signs of mucormycosis (Figure 1.3) vary from non-ulcerated, hairless nodules (<10 mm in diameter), or plaques (10-54 mm diameter) that on occasion contain exuding purulent material (Connolly and Obendorf, 1998; Connolly *et al.*, 2001), to cutaneous ulcers (5-100 mm in diameter) (Munday *et al.*, 1998; Connolly *et al.*, 2001). These ulcers can progress to involve underlying muscle up to a depth of 10 mm below the skin, and lesions are sometimes found in the internal organs, particularly the lungs (Munday *et al.*, 1998; Connolly *et al.*, 2001; Stewart and Munday, 2005). The most commonly affected sites are the hindlimbs and tail (Connolly and Obendorf, 1998;

Stewart, 2001). The webbing of the front feet, trunk, head and bill are less commonly affected (Connolly and Obendorf, 1998).

Active lesions of mucormycosis appear to cause discomfort, and affected animals frequently scratch and rub against objects (Munday *et al.*, 1998). The ulcers are assumed to lead to impaired thermoregulation (due to loss of fur) and impaired mobility (Connolly and Obendorf, 1998). It is likely to predispose affected animals to secondary infections and flystrike (Connolly and Obendorf, 1998; Munday *et al.*, 1998). Death is known to occur as a result of mucormycosis, but mortality rates are difficult to determine due to the cryptic nature of the animal (Munday and Peel, 1983; Obendorf *et al.*, 1993; Connolly and Obendorf, 1998; Munday *et al.*, 1998).

Figure 1.3. Cutaneous lesions of mucormycosis. **Photos: Joanne Connolly.**

The first reported cases of mucormycosis occurred in 1982 (Munday and Peel, 1983) and, although its emergence may be in part a result of anthropogenic factors, in particular the possible introduction of *M.* amphibiorum to Tasmania via frogs in fruit consignments from Queensland (Munday *et al.*, 1998; Stewart and Munday, 2005), the disease now appears to be established in certain waterways in Tasmania (Gust and

Griffiths, 2011). Detailed reports have been made of mucormycosis - diagnosed using culture with mating experiments, clinical signs or histology – only in waterways which ultimately drain into the Tamar River in northern Tasmania (Munday and Peel, 1983; Obendorf *et al.*, 1993; Connolly and Obendorf, 1998; Munday *et al.*, 1998; Stewart, 2001). Macgregor *et al.* (2010) found no evidence of mucormycosis in 23 platypuses captured in the Inglis catchment in 2007-2008.

The long-term impact of mucormycosis on platypus populations is also still unknown (Gust *et al.*, 2009), but the disease is considered a conservation threat (Munday *et al.*, 1998). It is the only infectious disease known to cause significant morbidity and mortality in platypuses (Connolly and Obendorf, 1998). One previously ulcerated animal has been observed in good health on recapture (Stewart, 2001). However, "there are few reported cases of recovery from the disease" (Stewart and Munday, 2005, p.128). Separate studies at two different sites both observed prevalences of 36% over 12 months (Connolly and Obendorf, 1998; Stewart, 2001). Both Stewart (2001) and Gust *et al.* (2009) demonstrated a reduction in prevalence at infected sites over periods of years. However, despite the low observed disease prevalence in affected catchments, Gust and Griffiths (2011) reported that the proportion of juveniles captured in these areas was twice as high as that in other catchments which may indicate significant population effects of the disease.

1.4.3 Other reported infections of platypuses

A range of other infections have been reported in platypuses, but there have been only a few documented cases of significant associated disease. The infections of platypuses and their effects are summarised in Table 1.2.

Table 1.2. Infections reported in platypuses and their effects.

Infectious Agent	Disease in Platypus	Source
Viruses		
Adenomavirus-like agent	Minor lesions in kidneys	Whittington *et al.* (1990)
Papilloma virus (possible diagnosis on basis of clinical signs)	Papules on webbing of feet	Munday *et al.* (1998)
Bacteria		
Leptospira spp.	Unknown, but seroconversion has been observed and spiral bacteria have been demonstrated in the renal cortex of a platypus that drowned in a fishing net.	Munday *et al.* (1998); Loewenstein *et al.* (2008)
Salmonella spp.	Diarrhoea in one case, systemic disease in two cases.	Munday *et al.* (1998)
Corynebacterium ulcerans	Cutaneous ulcer	Macgregor *et al.* (2010)
Widespread bacteria	Possibly secondary infections or contaminants	Whittington and McColl (1983); Munday *et al.* (1998)
Fungi		
Mucor amphibiorum	Mucormycosis – lesions in skin and sometimes internal organs. May result in death	Munday and Peel (1983); Obendorf *et al.* (1993); Connolly and Obendorf (1998); Munday *et al.* (1998); Stewart (2001)
Trichophyton mentagrophytes var *mentagrophytes*	Alopecia of the tail	Whittington (1992) cited by Munday *et al.* (1998)
Unknown fungal organism	Cutaneous granuloma	Macgregor *et al.* (2010)
Protozoa		
Theileria ornithorhynchi	Usually no effect. Haemolytic anaemia has been observed in two heavily infected animals.	Collins *et al.* (1986); Kessel *et al.* (2014)
Trypanosoma binneyi	None observed	Munday *et al.* (1998); Paparini *et al.* (2014)
Toxoplasma gondii	None observed	McColl (1983)
Coccidia	None observed	Munday *et al.* (1998)
Trematodes		
Mehlisia ornithorhyni	None observed	McColl (1983);Whittington and Spratt (1989)
Maritrema ornithorhynchi	None observed	Munday *et al.* (1998)
Moreauia mirabilis	None observed	Munday *et al.* (1998)
Cestodes		
Spirametra erinacei	Focal pneumonia	Whittington *et al.* (1992)
Nematodes		
Rhabditoid & filarioid spp.	Mild tissue changes in skin	Spratt and Whittington (1989); Whittington and Spratt (1989)
Arthropods		
Pygiopsylla hopli	Skin irritation	Munday *et al.* (1998)
Pygiopsylla zethi	Skin irritation	Munday *et al.* (1998)
Trombiculid mites (2 species)	Skin irritation	Munday *et al.* (1998)
Ixodes ornithorhynchi	Mild, chronic dermatitis	McColl (1983); Whittington and Spratt (1989); Munday *et al.* (1998)

1.5 AIMS OF THE PROJECT

The overall aims of the project were to develop and implement a framework to assess the health of wild platypuses and the ability of platypus populations to remain viable in the long term, for which the terms 'population health' or 'health of a population' are used interchangeably. A holistic approach to population health was taken, in part in recognition of the wide range of factors with the potential to affect the conservation status of the platypus and in part to mitigate the effects on the assessment process of the difficulties in assessing platypus population size. Data was gathered on individual platypus health, consistent with the increasing recognition of disease as an indicator and a cause of population declines in wildlife species (Deem *et al.*, 2001; Munson and Karesh, 2002; Tompkins and Jakob-Hoff, 2011). A wide range of more traditional ecological factors such as habitat characteristics, population age/sex structure and distribution, short and medium term movements, reproduction, survivorship and measures of population density were also investigated. Lastly immunogenetic diversity was assessed as a measure of the likely ability of populations to respond to new infectious challenges (Lillie *et al.*, 2012) and also as a guide to long term movements within and between populations (Kolomyjec *et al.*, 2009).

In order to overcome, as far as possible, the effects of migration of individuals into and out of a study area on the study's results, the population health assessment framework assumed that the smallest population that was studied was all the platypuses in a connected system of water bodies – i.e. all the platypuses within a single river catchment. Although platypuses are known to move over land, their usual mode of

travel is in water and their migration rate between catchments is likely to be much lower than that within catchments (Munday *et al.*, 1998; Otley *et al.*, 2000).

Rather than categorising or scoring the health of a population, the output of the health assessment was intended to be descriptive in nature. This approach is consistent with the current approaches to health monitoring in other wildlife species (Leendertz *et al.*, 2006; Vaughan, 2008; Schwacke *et al.*, 2010). It is also consistent with the approach taken in the field of human population health, although the latter has a definition of population health focused on individual well-being rather than long-term maintenance of population size (Kindig and Stoddart, 2003). It was intended that the comprehensive data gathered would allow identification of environmental, temporal and individual patterns of health parameters within populations, as well as comparisons between populations to help guide future research and conservation management plans (Kindig and Stoddart, 2003; McDowell *et al.*, 2004; Leendertz *et al.*, 2006; Schwacke *et al.*, 2010).

The project also had two secondary aims. Firstly, the development of three novel techniques was required: 1) the use of in-stream microchip readers for remote monitoring of platypuses, 2) the use of ultrasonography to assess the internal reproductive organs and 3) the use of ultrasonography to assess the amount of fat stored in a platypus's tail. Secondly, the project aimed to provide baseline health information and significantly contribute to the published literature on a range of factors related to platypus health and conservation. Due to the extensive nature of clinical examinations and sample collection and the need to reduce stress on the animals, the development of

protocols for the safe field anaesthesia of platypuses in the cold climate of Tasmania was necessary.

1.6 ORGANISATION OF CHAPTERS

Chapters 2, 3, 4 and 7 of this thesis each address one component of the population health assessment framework. Chapters 5 and 6 are investigations into subjects for which background information, not provided by previous research, was required for the population health assessment framework to be implemented. Chapter 2 describes the numbers, locations and demographics of captured platypuses. Population density is investigated using the results of a survey of public sightings. In addition, the effect of broad scale habitat factors on capture rates is investigated and estimates are made of the sizes of the population in the two river catchments studied. In addition, Chapter 2 investigates the demographics of the study population and describes the general methods of the live capture/release fieldwork that is referred to throughout the thesis. Chapter 3 describes the use of in-stream microchip readers to remotely monitor platypus behaviour, continued presence in an area and survivorship. There is particular emphasis on behaviour in the breeding season. Chapter 4 investigates the diversity and distribution of Major Histocompatibility Complex Class II DZB alleles in the Seabrook Creek catchment. Chapter 5 describes endocrine and ultrasonographic investigations into the timing of the breeding season in the study area. Chapter 6 discusses the assessment of platypus body condition and compares the novel use of ultrosonographic tail fat imaging and potential new body condition indices based on standard body measurements with the two previously reported measures. Chapter 7 focusses on the health assessment of the individual captured platypuses using previously reported methods and reports serum biochemistry and haematology reference ranges for the

study population. The thesis is summarised in the last chapter (General Discussion), which brings together the findings of the experimental work. This chapter gives an assessment of the health of the study populations, describes the implications of this study of platypus conservation management plans, provides a summary of the population health assessment framework and makes suggestions for future research.

Chapter 2.

Population demographics

2.1 INTRODUCTION

Age structure, adult sex ratio and geographical distribution are all factors that can influence the long-term viability of a wildlife population. In some species, adult sex ratios are known to naturally vary from parity, driven by factors such as mating systems, parental care, individual behaviour, migratory strategy or lifetime productivity (Ewen *et al.*, 2001; Donald, 2007). However, in other species, deviations from a 1:1 sex ratio have been associated with population declines in the wild (Wilkinson *et al.*, 2002; Donald, 2007), with populations surviving in degraded habitats (Martin and Handasyde, 2007; Flynn *et al.*, 2011) or as a result of inbreeding depression in captivity (Wilmer *et al.*, 1993; Sheffer *et al.*, 1999). Changes in age structures of populations can be an indication of negative impacts on a population (Bodkin *et al.*, 2000; Hutchings, 2005). Consistent with one of the manifestations of extinction debt, a concept that refers to the delayed impact on population numbers following environmental change, in longer lived wildlife species changes to age structures often occur more quickly than changes in population size (Tilman *et al.*, 1994; Doak and Morris, 1999; Holmes and York, 2003; Hylander and Ehrlén, 2013). Changes in the geographic distribution of a species is a common feature of declining wildlife populations (Fuller *et al.*, 1995; Robinson *et al.*, 1995; Rodríguez, 2002). Fragmented populations are likely to undergo further declines due to reduced availability of mates, reduced replenishment of numbers by migration, and inbreeding depression (Hylander and Ehrlén, 2013).

However, in many situations, age structure, adult sex ratio and geographical distribution of wildlife populations are considered in relative isolation and their meanings can be unclear. Collection of data on these factors alongside data on individual health, movements and survivorship as well as population genetic diversity, and habitat factors

should allow better interpretation of results and is an important aspect of the population health assessment framework developed in this thesis.

In platypus populations, a range of sex ratios in study populations has been reported. For example, the two largest studies have reported biases towards different sexes. Grant (2004) observed an adult sex ratio (female:male) of 1.65:1 from a total 469 adults captured over 30 years in the Upper Shoalhaven River, New South Wales. Serena and Williams (2012) observed a sex ratio of 0.79:1 from 1326 adult/subadults captured in 15 river basins across Victoria over 15 years. The proportions of captured individuals that have been juveniles have also varied. Grant (2004) reported that 33% of individuals captured were juveniles; while Serena and Williams (2012) reported that 19% of total captures/recaptures were juveniles. Studies in Tasmanian populations have showed juvenile rates of 8-15% (Bethge, 2002; Koch *et al.*, 2006; Gust and Griffiths, 2011). Gust and Griffiths (2011) observed a higher proportion of juveniles captured in a river catchment affected by the fungal disease mucormycosis, suggesting a reduced adult survival rate due to the disease. Koch *et al.* (2006) observed a higher proportion of juveniles amongst platypuses captured in headwater streams, indicating a link between environmental factors and local age structure.

Estimation of platypus population size/density has rarely been attempted. Serena (1994) made population estimates of 1.3-2.1 platypuses per km of a creek with an average width of 3m and maximum depth of 1.4m. Grant and Carrick (1978) estimated that a 1.8km stretch (consisting of a 940m pool and a 500m pool separated by a series of riffles) of the Shoalhaven River in New South Wales supported a minimum population of 14-18 platypuses. Furlan *et al.* (2012) estimated that there were ~110 in the

introduced population of platypuses on Kangaroo Island, South Australia. Fox *et al.* (2004) created a metapopulation model for the platypus in northeast Tasmania but noted that there was a lack of certainty about the information and assumptions put into the model.

Surveys of public sightings of platypuses have been used to investigate platypus population distribution and density, as well as the impacts of potential threats they face (Grant and Llewellyn, 1991; Connolly and Obendorf, 1998; Turnbull, 1998; Rohweder and Baverstock, 1999; Otley, 2001; Lunney *et al.*, 2004). Lunney *et al.* (2004) found that the distribution of platypus sightings recorded in a public survey corresponded closely with field observations and captures. Grant and Llewellyn (1991) attempted to investigate population density by asking respondents to categorize platypus sightings at locations they were familiar with as absent, rare, common or abundant. It was reported that respondents found it difficult to distinguish between the common and the abundant categories (Grant and Llewellyn, 1991).

Platypuses are known to exist in the majority of freshwater systems in Tasmania. Although different capture methods are used in different types of water body, capture rates in lotic systems are generally greatest in streams of higher order as defined by Strahler (1957) (Connolly *et al.*, 1998; Koch *et al.*, 2006; Olssen-Herrin, 2009), and capture rates in lakes and farm dams have also been relatively high (Bethge, 2002; Olssen-Herrin, 2009). The habitat variables that are generally considered to be the most important to the platypus are those associated with access to earth banks consolidated with roots of vegetation for burrow construction, and those associated with availability of benthic invertebrate food species (Rohweder, 1992; Grant and Bishop, 1998).

Variables which fit one or both of these categories and that have been observed to be positively related to platypus occurrence are area, height and slope of consolidated earth banks, tree density on banks, proportion of banks overhung by vegetation, proximity of water to consolidated earth banks, pool depth within a certain range, pool length, benthic production index and the permanence of water body (Woon, 1995; Ellem *et al.*, 1998; Grant and Bishop, 1998; Turnbull, 1998).

However, observations of platypus burrows in piles of vegetation, soil and debris in the stream channels led Serena *et al.* (1998) to suggest that the habitat requirements for burrow construction may not be as strict as previously thought. Burrows have more recently been found in sedge tussocks and in stream caves (Otley *et al.*, 2000; Munks *et al.*, 2004). Woon (1995) recognised that "it may not be possible to isolate specific habitat preferences since the habitat variables represent a complex system of interrelated habitat characteristics".

This study investigates the distribution, density, age structure and sex ratio of the study populations. Secondary aims were to investigate factors that may affect platypus population density and distribution, and to develop a novel method of using the data collected to produce measures of population size.

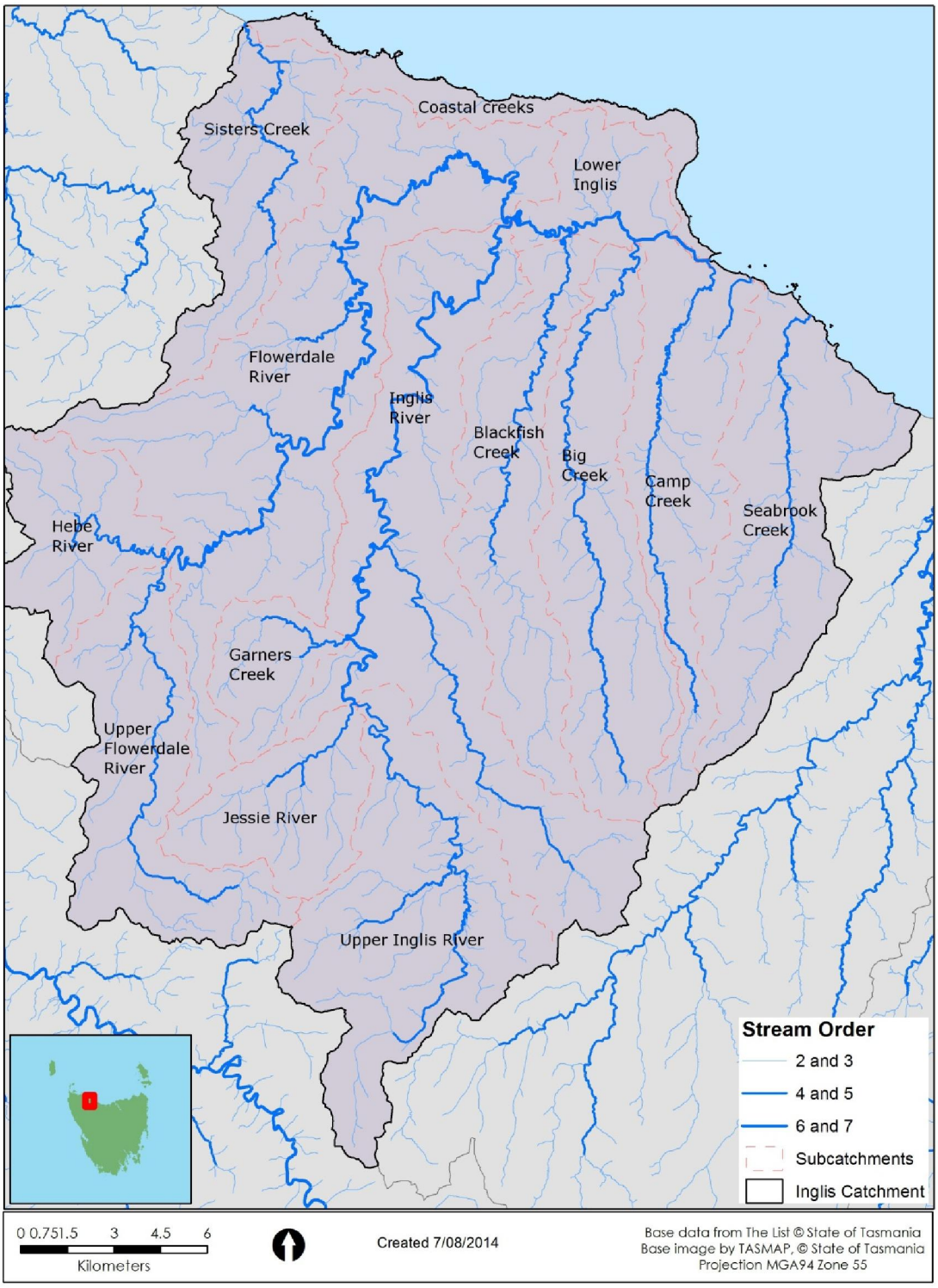
2.2 METHODS

2.2.1 Study area

The fieldwork for this study was performed in the Inglis Catchment (41.06°S, 145.64°E) in northwest Tasmania, Australia (Fig 2.1). For the purposes of management and administration, Tasmania is divided into 48 areas known as Comprehensive Freshwater

Ecosystem Values catchment areas (CFEV database, 2005). Most of these are dominated by one large river basin, but also contain other smaller hydrologically distinct areas. The majority of the Inglis Catchment drains into the sea via the Inglis River at Wynyard. For the rest of this thesis, this area will be referred to as the Inglis River Catchment. The Comprehensive Freshwater Ecosystem Values database (2005) divides this part of the catchment into ten subcatchments, the largest two of which are drained by the Inglis and Flowerdale Rivers, which join 10.9 km before flowing into the Bass Strait at Wynyard. Prior to their confluence the Inglis River is approximately 31.4 km in length and the Flowerdale River is approximately 42.6 km in length (DPIPWE, 2007). Names were given to the subcatchments of the Inglis River Catchment for the purposes of this project (Figure 2.1). For this project, the area including the Inglis River below the confluence of the Inglis and Flowerdale Rivers as well as the creeks flowing into it from the north were considered to be in an eleventh subcatchment, the 'Lower Inglis River Subcatchment', and not part of the Blackfish Creek Subcatchment as defined by CFEV database (2005). There are three other areas in the Inglis Catchment that do not lead into the main Inglis/Flowerdale River system (CFEV database, 2005). To the west and east are areas drained by Sisters Creek and Seabrook Creek, respectively (CFEV database, 2005; DPIPWE, 2007). Along the coast to the north is an area containing a number of small creeks that lead directly into the sea (Figure 2.1). Of these, only the area containing Seabrook Creek will be mentioned again during this thesis, and it will be referred to as the Seabrook Creek Catchment.

Figure 2.1. Location of the Inglis Catchment, in Tasmania, and the subcatchments within it (named for this project). Stream orders as described by Strahler (1957).

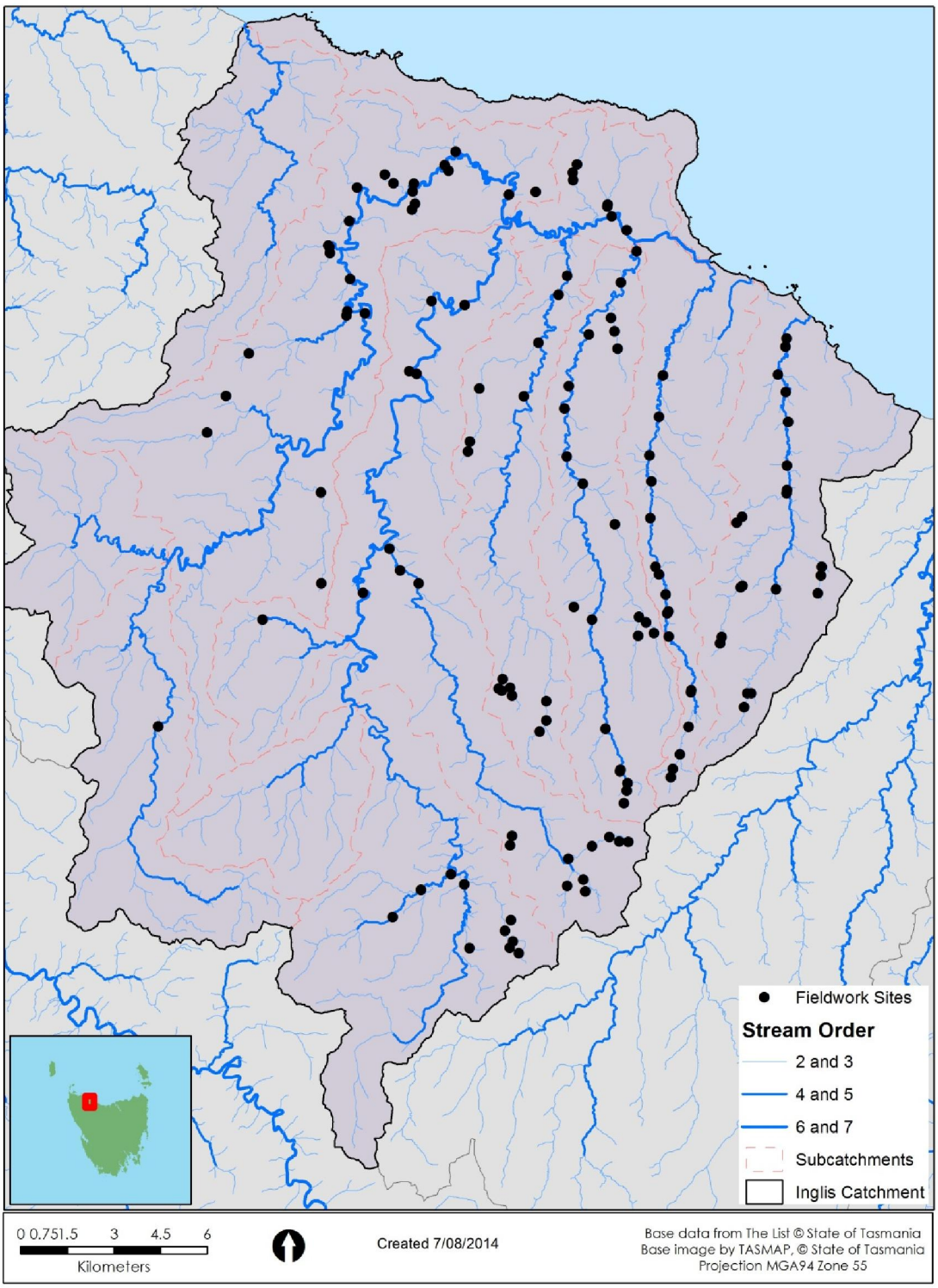


The Inglis catchment covers an area of 615 km². The average annual rainfall varies from around 1,000 mm on the coast to over 1,600 mm in the upper catchment (DPIPWE, 2007). There is a gradient of land-use across the catchment with forestry dominating to the west and south, and agriculture dominating to the east and north (DPIPWE, 2007). Due to the steep topography around the upper Inglis and Flowerdale Rivers, substantial amounts of native forest have been retained in these areas (DPIPWE, 2007). Urban activities are common around the river estuary as it runs through the town of Wynyard (Figure 2.2).

Figure 2.2. Satellite image of the study area, created at www.thelistmap.tas.gov.au, **showing forested (dark green) areas and urban areas (red).**

2.2.2 Site selection

Figure 2.3. Locations of fieldwork sites



One hundred and thirty eight fieldwork sites were selected within the study area (Figure 2.3). Of these sites, 19 had been used for platypus capture fieldwork in a smaller study in 2007-2008 (Macgregor *et al.*, 2010). Sites were selected 1) to provide a broad geographical coverage of the study area, 2) to represent the major land-uses in the catchment, 3) only if they were suitable for the capture of platypuses with fyke nets, 4) only if there was reasonable vehicular access, and 5) only if permission of the land owner was granted to access the property. Overall, sites were selected in nine of the eleven subcatchments in the Inglis River Catchment, as well as the Seabrook Creek Catchment to the east. The Hebe River and Jessie River Subcatchments were excluded due to lack of vehicular access. Vehicular access was also poor in the Upper Flowerdale and Garners Creek Subcatchments. Sites were considered separate when they were in a different river/creek or were in the same river/creek but greater than 500m apart or separated by a farm dam. The numbers of sites in each subcatchment are listed in Table 2.1.

Table 2.1. Number of fieldwork sites in each subcatchment.

Subcatchment	Number of fieldwork sites
Lower Inglis River	7
Flowerdale River	25
Inglis River	18
Blackfish Creek	15
Big Creek	22
Camp Creek	18
Upper Inglis	10
Garners Creek	1
Upper Flowerdale	1
Seabrook Creek	21

The schedule of fieldwork at these sites was not fixed but was determined on an ongoing basis with the following priorities listed in descending order:

1) Maximising the number of platypuses captured during the study

2) Having platypus captures evenly distributed between spring (September to November), summer (December to February), autumn (March to May) and winter (June to August).
3) Having captures in all subcatchments and distributed evenly between spring, summer, autumn and winter within each subcatchment as far as possible.
4) Having fieldwork sessions at upper, middle and lower levels of the catchment and in different land-use areas in all seasons.

The fieldwork schedule was also affected by:

1) Periods of flood or low water flow.
2) Access to fieldwork sites, particularly resulting from stock rotation for sites on agricultural land.
3) Availability of fieldwork volunteers.

2.2.3 Capture methods

Platypuses were captured using fyke nets as described by Serena (1994). Nets were generally set mid to late afternoon (range 14:30h-20:45h) and removed from the water around 23:00h to 24:00h (range 19:15h-01:45h) that night.

Fyke nets were set in pairs in rivers/creeks with one net facing upstream and the other facing downstream in close proximity in order to allow platypuses moving in either direction to be captured. The stream width at these sites was between 40 cm and 12 m and the depth was usually less than 50 cm but was occasionally up to ~1 m in places (Figure 2.4 and Appendix A). On two occasions nets were set in dry riverbeds. On one occasion they were set across a track on land between two farm dams where footprints and drag marks consistent with platypus movement were present. To allow a trapped

platypus access to air to breathe, each fyke net was set so that at least one third of it remained above the water level along its full length. Also, the distal end of each fyke net was staked completely above the water level. Fyke nets were checked every 30-60min.

Figure 2.4. Fyke nets set in streams of different sizes **(Photos a: Helen Robertson, c: Adrian Miller).**

Platypuses were removed from the nets as soon as they were found. Where possible the distal end of the net was untied and placed in a cotton sack and the platypus was encouraged to move along the net and into the sack. If the terrain at the fieldwork site did not allow this, or if a platypus was reluctant to move into the sack, it was removed from the net by hand and placed in the cotton sack (Figure 2.5). Any time a conscious platypus had to be picked up during this project great care was taken to avoid

envenomation by the spurs on the hindlimb of the males. Even when the platypus was known to be a female, the same procedure was followed. The first step in picking up a platypus was to discourage it from moving by placing a hand on its back. Although this sometimes took a few attempts, platypuses would generally stay still when gentle pressure was applied to their back. The tail would then be grasped half way along its length with fingers curled around the dorsal surface and thumb along the ventral surface. The platypus would then be gently lifted upwards by the tail at the same time as the pressure on its back was released (Figures 2.5 & 2.6). The platypus was then held away from the handler's body and could be placed from above into a sack and the tail let go. Once the platypus was in the cotton holding sack, the sack was then tied closed with string and placed into a tied hessian sack.

Figure 2.5. (a-c) Encouraging a platypus (*Ornithorhynchus anatinus*) to move from a fyke net into a holding sack (photos: Deb Winfield), and (d-e) manually transferring a platypus from a fyke net into a holding sack (photos: David McArtor).

Figure 2.6. Correct hand position for platypus handling

Platypuses were held and examined near to their site of capture in the field. Where possible, platypuses in their holding sacks were taken to the holding/examination site on foot. When the capture site and holding/examination sites were more than ~500 m apart, platypuses were transported by car. The double bagged platypus was then kept in a quiet place with adequate protection from adverse weather conditions. Depending on the weather and the nature of the fieldwork site, this could be in a vehicle, in a farm building or in a sheltered place outside. This procedure kept the platypuses secure,

comfortable, dry and at a suitable temperature and also minimised stress. Each platypus was held in the sacks for at least 60 min to allow its fur to dry and to allow the stomach to empty prior to anaesthesia. The platypus has a fast throughput of ingesta through the gastrointestinal tract and 60 min is considered adequate for stomach emptying (Booth and Connolly, 2008).

Holding sacks were used no more than once per fieldwork session. Used sacks were washed, soaked overnight in bleach and dried in sunlight before re-use. After each fieldwork session debris was removed and the fyke nets they were rinsed in water. Additionally, after five uses or between uses in different river catchments, each net was soaked overnight in bleach then dried in sunlight.

Holding, handling and anaesthesia protocols were changed after the first platypus capture, in which the platypus became hypothermic and had an extended recovery time. Details of the anaesthetic procedure used for the first platypus are given in Appendix B. The remainder of the protocols in Sections 2.2.3 and 2.2.4 apply to platypus 2 onwards.

When ambient temperature was <10°C, a hot water bottle containing water at 34°C was placed between the hessian and cotton holding sacks in a position where it was not in contact with the platypus, but where the platypus could move towards it if it chose to.

2.2.4 Anaesthesia

Platypuses were examined under anaesthesia to reduce stress. Anaesthesia and examination took place in the field close to the capture site(s) either in a three sided tent (Figure 2.7) or in a farm building where one was available. For induction of anaesthesia,

each platypus was placed into a cotton sack with a hole ~5 cm long in one corner. The platypus was encouraged to put its bill through the hole. The loose parts of the cotton sack were gathered up by hand either side of the platypus and gentle pressure applied to restrain the platypus's body in the bag. A face mask made for this project was placed over the platypus's bill. Anaesthesia was induced with 5% isoflurane delivered by face mask in oxygen at a flow rate of 2 L/min. An adequate level of anaesthesia was usually achieved in 1-4 min and the isoflurane level was then turned down either gradually or in one step to the maintenance concentration. In most platypuses anaesthesia was maintained with 1.5% isoflurane delivered by face mask in oxygen at 1.5 L/min. The anaesthetised platypus was removed from the cotton sack and laid on a thermostatically controlled heat pad (ICU patient warming pad, Vetquip, Sydney, NSW, Australia) with a bubble wrap blanket placed over it. Heart rate, respiratory rate, body temperature and anaesthetic depth were assessed and recorded every 5 min. Respiratory rate was measured visually. Core body temperature was measured using a SureTemp® Plus 692 electronic thermometer (Welch Allyn®, Skaneateles Falls, NY, USA). Heart rate was measured by ausculatation. Pulse oximetry using a Nellcor N-65 pulse oximeter (Covidien plc., Dublin, Ireland) with the probe on the foot webbing or bill shield was used with minimal success. Doppler ultrasound using a Parks model 811-b doppler ultrasound (Parks Medical Electronics, Inc, Aloha, OR USA), with the probe placed in the midline of the ventral aspect of the tail, was successfully used in the last ten platypuses anaesthetised. The heat pad temperature was adjusted depending on the temperature of the platypus. A temperature of 34°C was found to be suitable for most platypuses. Anaesthetic face masks and the heat pad were disinfected between platypuses using F10SC (Health and Hygiene Pty Ltd, Sunninghill, South Africa). For

further details of anaesthetic protocols and observations, see Appendices B and C (Macgregor *et al.*, 2014).

Figure 2.7. Platypus field anaesthesia. a) Three sided examination tent, b) monitoring heart rate and pulse oximetry, platypus on thermostatically controlled heat pad, c) administration of isoflurane in oxygen via face mask, d) cloacal thermometer, e) platypus under bubble wrap blanket, f) position of Doppler ultrasound probe on ventral aspect of the tail. **Photos: Vincent Beaumont, Geoff Dutton, Deb Winfield and Yolande Szekfy.**

2.2.5 Examination and sampling

Body weight was measured before anaesthesia (±0.01kg; digital Rapala® scales) (Figure 2.8a) and then the following physical characteristics were recorded for each platypus under anaesthesia (Figure 2.8 b-e):

1) Sex and age by presence and morphology of spur (Figure 2.9) using the method described by Temple-Smith (1973) adjusted in accordance with the findings of Williams *et al.* (2013). Males were classified into the following age categories: juvenile (<1 year of age; full spur sheath present January –June, full or partial spur sheath present July- December), subadult (12-18 months of age; partial spur sheath present January- June), adult (>~15months; spur sheath absent). Females were classified into the following age categories: juvenile (<12 months; spur sheath retained March-December), subadult (spur sheath retained January-February) and adult (>9 months; spur sheath absent). Note that because of variable times of spur sheath loss, there is overlap between the age categories. Because Williams *et al.* (2013) noted that it is possible to misclassify a platypus's age class using this method in a small proportion of non-adult individuals, additional confidence in the results was provided by considering their body mass/size measurements.
2) Tail Volume Index as described by Grant and Carrick (1978) (1 = very good, 5 = very poor).
3) Spur length and width (mm) where present were assessed visually to minimise risk of envenomation.
4) Moult class as described by Grant and Carrick (1978).
5) Bill width (mm) at its widest point was assessed using vernier calipers.
6) Bill length without shield (mm) was assessed using vernier calipers.

7) Bill length including shield (mm) was assessed using vernier calipers.
8) Total Body length (cm) using a tape measure (tip of bill to tip of tail, measured over dorsum).
9) Tail length (mm) - distance from the tip of the tail (not including length of hair cover) to the caudal muscles of the body.
10) Tail width and depth (distance between dorsal and ventral surfaces) (mm) at the midpoint of its length was assessed using Vernier calipers.

Each platypus was checked for the presence of a microchip using a hand held Trovan® GR251 Universal Reader. Where no microchip was present, a Trovan Unique® microchip was implanted aseptically into the subcutaneous tissues between the scapulae of the platypus, using the methods of Grant and Whittington (1991), to allow subsequent identification of each individual (Figure 2.8f). If a microchip was present, its number was recorded.

Figure 2.8. Platypus examination a) weighing, b) measuring bill width, c) measuring bill length without shield, d) measuring bill length with shield, e) measuring total body length, f) inserting microchip. **Photos: Christina Shaw and Yolande Szekfy**

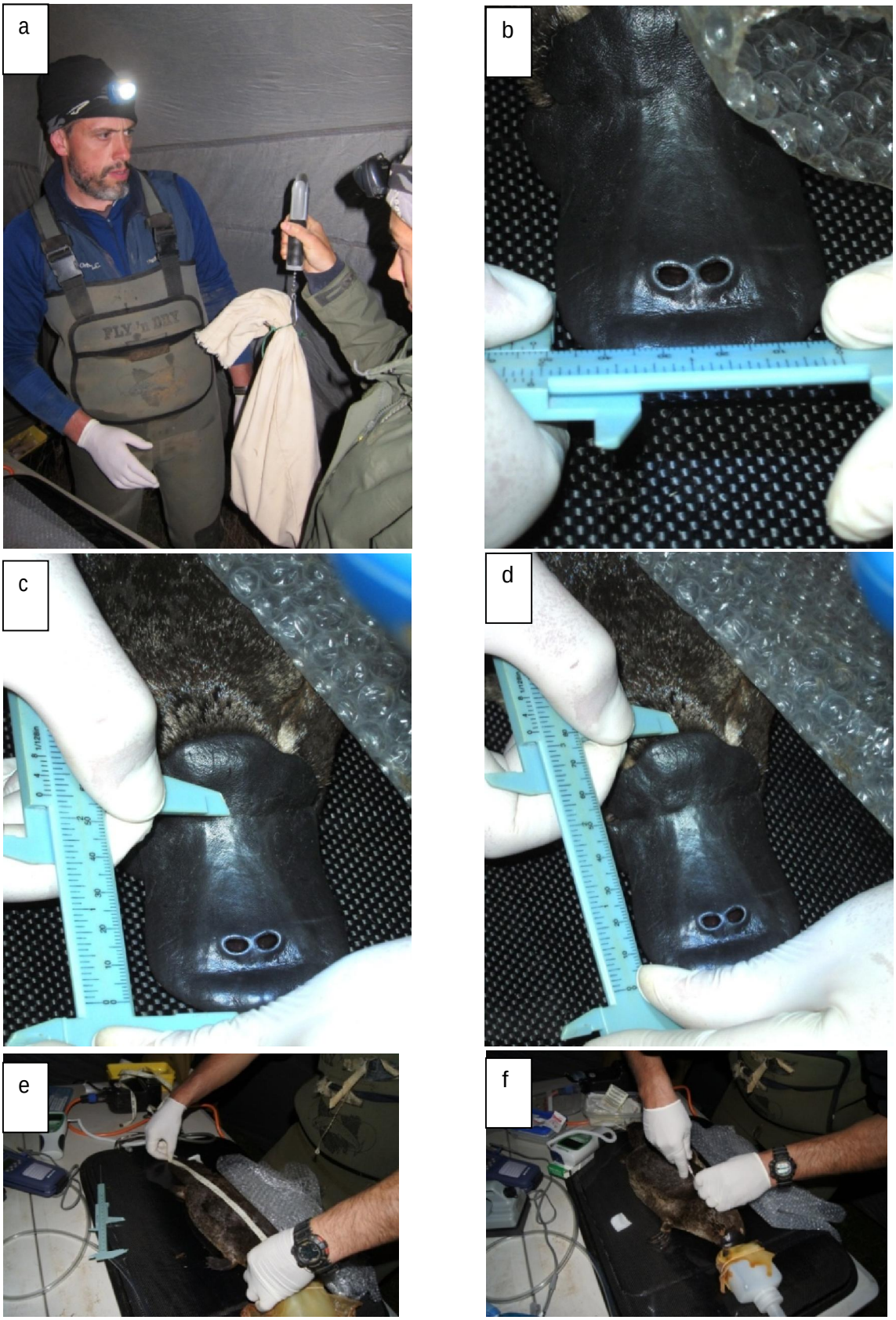
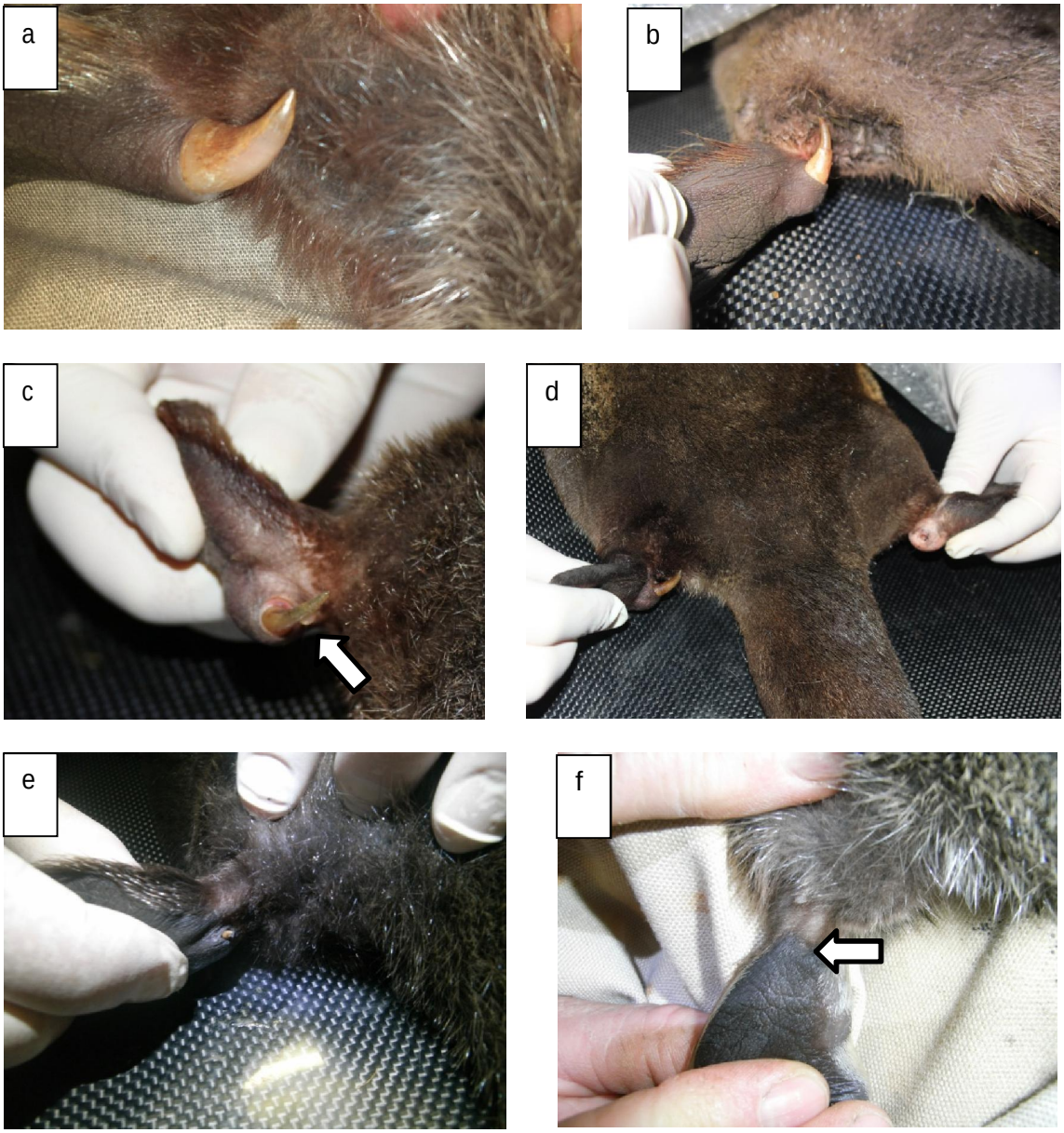


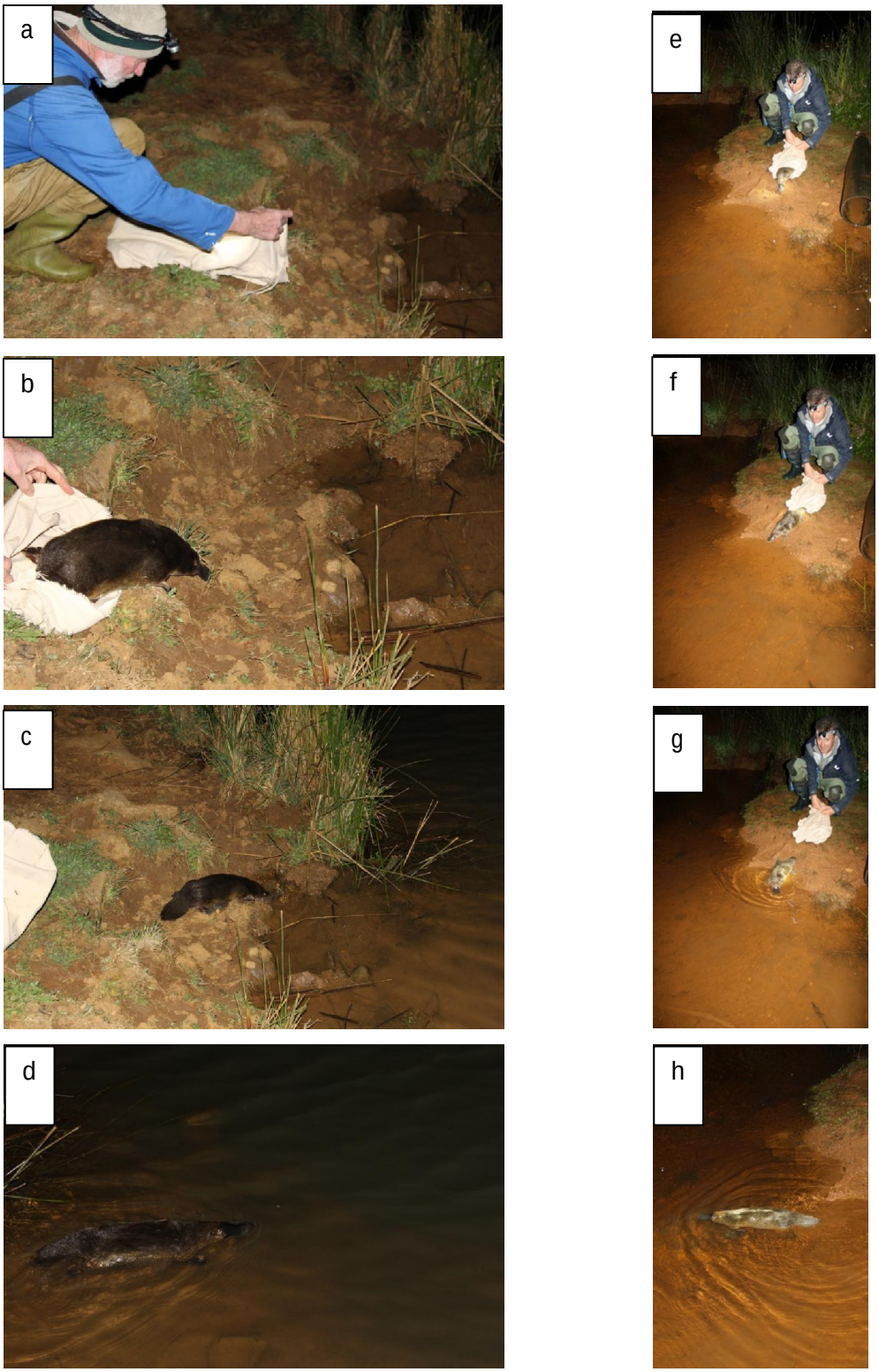
Figure 2.9. Platypus spurs/spur sheaths. a) male spur almost entirely covered by spur sheath, b) male spur half covered by spur sheath, c) adult spur with drop of venom (arrow), d) adult male with right spur lost, e) juvenile female with spur sheath present, f) adult female with depression where spur sheath has been lost (arrow). **Photos: Helen Robertson.**



2.2.6 Recovery and release

When monitoring led to concern about the condition of the platypus under anaesthesia, some or all of the remaining procedures were omitted. In all individuals, recovery from anaesthesia was started by turning off isoflurane delivery and allowing the platypus to breathe oxygen delivered by face mask or air. Isoflurane administration was not recommenced after the face mask had been removed for blood sampling, which when performed was the last procedure, and the platypus was allowed to recover as above. A platypus was considered to be conscious again when its eyes opened, at which point it was then transferred to a dry cotton sack and hessian sack. If the ambient temperature was below 10°C and/or the platypus's body temperature was low at the end of anaesthesia, a hot water bottle at 32-34°C was placed between the holding sacks as before the anaesthetic procedure. Each platypus was checked briefly at least once after recovery. The exact number of checks depended on the progress of the recovery but was kept to a minimum to reduce stress. If the platypus moved when the sack was approached or gently touched no further checking was needed. Otherwise the sacks would be opened to check for respiration, movement, alertness and heart rate if indicated. Each platypus was held for a minimum of 60mins before release at the site of its capture (Figure 2.10).

Figure 2.10. Platypus release (a-d & e-h). **Photos: Helen Robertson and Geoff Dutton.**



2.2.7 Survey of public sightings

A survey of public sightings was performed between 1st January 2013 and 30th April 2013. The survey was publicised in local radio interviews and newspaper articles in which contact details were given so that any interested members of the public could request and receive a hard or electronic copy of a questionnaire (See Appendix D). The questionnaire was also distributed from Wynyard Veterinary Clinic in Wynyard, the largest town in the study area. Members of the public were also made aware of the public survey and the overall platypus research project at a stall at the Wynyard Agricultural Show on 18th March 2013 (Figure 2.11). Interested members of the public were asked to either complete a questionnaire and return it at the Show, or take a questionnaire and return it at a later date. Contact was also made with others in the local community who had expressed an interest in the platypus research, to ask them if they would like to complete a questionnaire.

The questionnaire asked respondents to list locations where they had seen platypuses. They were asked about their sightings of platypuses at each location during two time periods: a) 1st January 2012 to 31st December 2012, and b) before 1st January 2012. For each of these two time periods, respondents were asked to describe the frequency of their sightings in one of three ways: i) “Rare”, ii) “Common”, or iii) “Don’t know”. They were also asked to give an approximate date and time of day of sightings if known. In addition, respondents were asked to give details of waterbodies with which they were familiar where they had never seen a platypus.

Figure 2.11. Stall at the Wynyard Agricultural Show to publicise the survey of public sightings. **Photo: Helen Robertson.**

2.2.8 Fieldwork site characteristics

Broad habitat variables were chosen for their likely importance in determining habitat quality for platypuses and their inclusion in previous studies of platypus population density (Ellem *et al.*, 1998; Bethge, 2002; Koch *et al.*, 2006; Gust and Griffiths, 2011). The following characteristics were compiled for each fieldwork site:

1) Northings and Eastings using an eXplorist 210 handheld GPS device (Magellan, Santa Clara, CA, USA).
2) Subcatchment as described by CFEV database (2005) with one amendment (See Section 2.2.1).
3) Stream order (Strahler, 1957; CFEV database, 2005).
4) Altitude estimated from 10m gridlines – base data from theLIST© State of Tasmania (www.thelist.tas.gov.au).

5) Percentage of forest cover (including native and plantation) within a 500m radius of the site. These areas were measured on the topography basemap layer at theLIST, using the polygon area measurement tool available on that website. Satellite images available at https://maps.google.com.au, and my own knowledge of the fieldwork sites, were used to check that the areas of forest shown on theLIST, were up to date. The mean % of forest area with a 500m radius of fieldwork sites was 39%. The mean % forest area in the combined Inglis River and Seabrook Creek Catchments was 49%.
6) Total water surface area within 500m of the site along connected waterways. To estimate this parameter, the stream order of surrounding streams was determined using CFEV database (2005). The length of each stream order (1-7) within 500m by water was measured on the topography basemap theLIST©, using the distance measurement tool available on that website. The total surface area of dams was measured on the topography basemap layer at theLIST©, using the polygon area measurement tool available on that website. Satellite images available at https://maps.google.com.au, and my own knowledge of the fieldwork sites, were used to check that the man-made waterbodies shown on theLIST were up to date. The total surface area of rivers was estimated using the following equation, where SOx = Length of stream order x within 500m:
    a. River surface area within 500m = (0.5*SO1) + (1*SO2) + (2*SO3) + (4*SO4) + (6*SO5) + (10*SO6) + (20*SO7)
7) Total water surface area within 500 m was calculated by adding river surface area within 500 m to dam surface area within 500 m.
8) Total stream length within 500 m of connected waterway as described in 6).

9) Proportion of forest cover in the subcatchment containing the site (DPIPWE, 2009).

2.2.9 Capture effort/capture rates

Consistent with the findings of previous studies that few platypuses enter fyke nets during daylight (Serena and Williams, 2012), the definition of capture effort only included time after sunset, as follows: one net pair hour = one pair of nets, one net facing upstream one net facing downstream, in place at a single site for one hour after sunset. The capture efforts for each subcatchment are shown in Table 2.2. Capture rates were calculated as the number of platypuses captured / capture effort.

Table 2.2. Capture effort (net pair hours) by subcatchment and season.

Subcatchment	Spring	Summer	Autumn	Winter	**TOTAL**
Lower Inglis River	14.23	7.03	38.47	0.00	**59.73**
Flowerdale River	57.55	39.95	76.52	40.35	**214.37**
Inglis River	22.10	12.47	81.00	17.95	**133.52**
Blackfish Creek	23.03	18.85	38.37	48.02	**128.27**
Big Creek	41.52	33.90	50.77	52.48	**178.67**
Camp Creek	16.82	42.02	32.18	58.58	**149.60**
Upper Inglis	17.28	19.42	37.00	20.92	**94.62**
Garners Creek	0.00	3.98	5.47	0.00	**9.45**
Upper Flowerdale	0.00	3.65	5.00	0.00	**8.65**
Seabrook Creek	3.72	64.07	15.97	74.63	**158.38**
TOTAL	**196.25**	**245.33**	**380.73**	**312.93**	**1135.25**

2.2.10 Statistical analysis

The effect of site and repeated fieldwork sessions on the total number of platypuses captured was investigated using a mixed-model ANOVA (fieldwork site and session number for that site as random factors). The effect of habitat characteristics on capture success was also investigated. Because the number of fieldwork sessions at each site was not standardised (range 1-6), this was investigated for the first session at each site

only. Capture success for this analysis was measured by the number of platypuses captured in the first session at each site rather than by capture rates. Figures 3.9 and 3.10 indicate that platypus movements past the fieldwork sites (and hence capture opportunities at fieldwork sessions) mostly occurred between dusk and dawn regardless of the season, so this approach avoided the potential for the introduction of bias into analysis due to the effect of the seasonally varying number of night-time hours on capture rates (number of captures/number of hours nets in water after dusk; see Section 2.2.9). Forward stepwise regression was performed with the number of platypuses captured during the first fieldwork session at each fieldwork site as the dependent factor and four variables chosen to reflect site-level habitat characteristics (altitude, amount of forest cover within a 500 m radius, total water surface area within 500 m of connected waterway, river length within 500 m) and one variable chosen to represent the broader habitat characteristics of each subcatchment (proportion of forested land in the relevant subcatchment) as independent variables using Statistica 8.0 (Stat Soft Inc. Tulsa, OK, USA). Differences between seasons in the sex ratios of captures were tested using a χ^2 test (http://www.socscistatistics.com). The number of captures for each sub-catchment was tested against the average capture rate across all sub-catchments by χ^2 test. Captures were also compared across seasons by χ^2 test with expected values calculated assuming an equal proportion of animals captured each season (expected numbers calculated from the capture effort) and between the sexes assuming an equal proportion of captures of each sex. Additionally, the number of platypuses captured before and after sunset were compared with expected values assuming an equal probability of capture in these time periods, and the number of platypuses captured in each hour after sunset was compared with expected values assuming equal capture rates in each hour.

2.2.11 Population size

An estimate of the minimum number platypuses observable using the methods of this study was made for each of the two study populations (Inglis River Catchment and Seabrook Creek Catchment; See Figure 2.1) based on the following assumptions:

1) On average, platypuses with a stable home range use 1,000 m, in an upstream-downstream direction, of the river in which they live over each 24 h period, regardless of the width of that river or the presence of lakes or artificial dams. This is an approximate figure based on the findings of previous live capture and radiotracking studies (Carrick and Hughes, 1978; Serena, 1994; Gardner and Serena, 1995; Gust and Handasyde, 1995; Serena *et al.*, 1998; Serena and Williams, 2013).
2) The expected number of platypuses in a waterbody (either foraging in the water or resting in an adjacent burrow) at any point in time is directly related to the surface area of that water body.
3) The 138 fieldwork sites were representative of the two study catchments in terms of geographical distribution, hydrological characteristics, altitude and surrounding land-use practices.

Total water area within 500 m of connected waterway of the 138 fieldwork sites, total catchment river area and total catchment dam area (A, E and F, respectively, in Table 2.10) were calculated as in section 2.2.8. The minimum number of platypuses using the fieldwork sites during the course of the project (B in Table 2.10) was calculated using capture number data and data from the associated remote monitoring (see Chapter 3). Remote monitoring was only performed at 18 of the 138 fieldwork sites. Of the 12 platypuses captured in 2007-2008 at those sites where remote monitoring was

performed in 2012-2014 (Macgregor, 2008), five (42%) were still present at the sites of their captures during the course of this study (see Section 3.3). It was therefore assumed that 42% of the 23 platypuses captured in 2007-2008 at all the 138 fieldwork sites in this study were still present at the sites of their captures. This figure (23 x 0.42 = 10) was added to the total number of individual platypuses captured in this project to produce a figure for the minimum number of platypuses using the fieldwork sites during the course of the study (B in Table 2.10).

Section 3.3 suggests approximately 18% of the captured platypuses may be only transient users of the capture sites. Repeated fieldwork sessions would be expected to overestimate the number of platypuses using the fieldwork sites at any one time because of ongoing capture of new transient individuals. In order to avoid this, a correction was made that attempted to ensure that transient individuals from only one night per site were counted. It was assumed that transient captures at each site were evenly distributed between sessions. Because the mean number of sessions per site was 2.03, only half of the 18% of individuals assumed to be transient were to be counted. As a result, the correction made for transient individuals was that 9% of the capture numbers were subtracted from the total number of observable platypuses to produce a figure for the minimum number of platypuses using the fieldwork sites on any particular night (C in Table 2.10). This figure was divided by the total water surface area within 500 m of all the fieldwork sites to produce a figure for the mean density of the platypus population by water surface area (D in Table 2.10). The mean population density figure was multiplied by the total water surface area in each of the two study catchments (G in Table 2.10) to produce the minimum observable population size estimates (H in Table 2.10).

2.3 RESULTS

2.3.1 Locations, ages and sex of captured platypuses

A total of 154 individual platypuses (63 adult females, 3 juvenile females, 76 adult males, 6 subadult males, and 6 juvenile males) were captured across all study sites between 29/8/2011 and 31/8/2013 and there were 12 recaptures. One adult male was recaptured in Seabrook Creek 1.5 km upstream of its first capture site. No other platypuses were captured at more than one site. There were no recaptures of individuals captured and microchipped during previous fieldwork in 2007-2008. The locations/numbers of first captures are shown in Figure 2.12. Captures were well distributed through the catchment with the exception of the inaccessible forested areas to the southwest of the catchment where sampling could not be undertaken. The distribution of captures between subcatchments was proportional to the capture effort in each subcatchment (Table 2.3).

Figure 2.12. Total number of first captures at each fieldwork site. A-R = remote monitoring sites (Chapter 3).

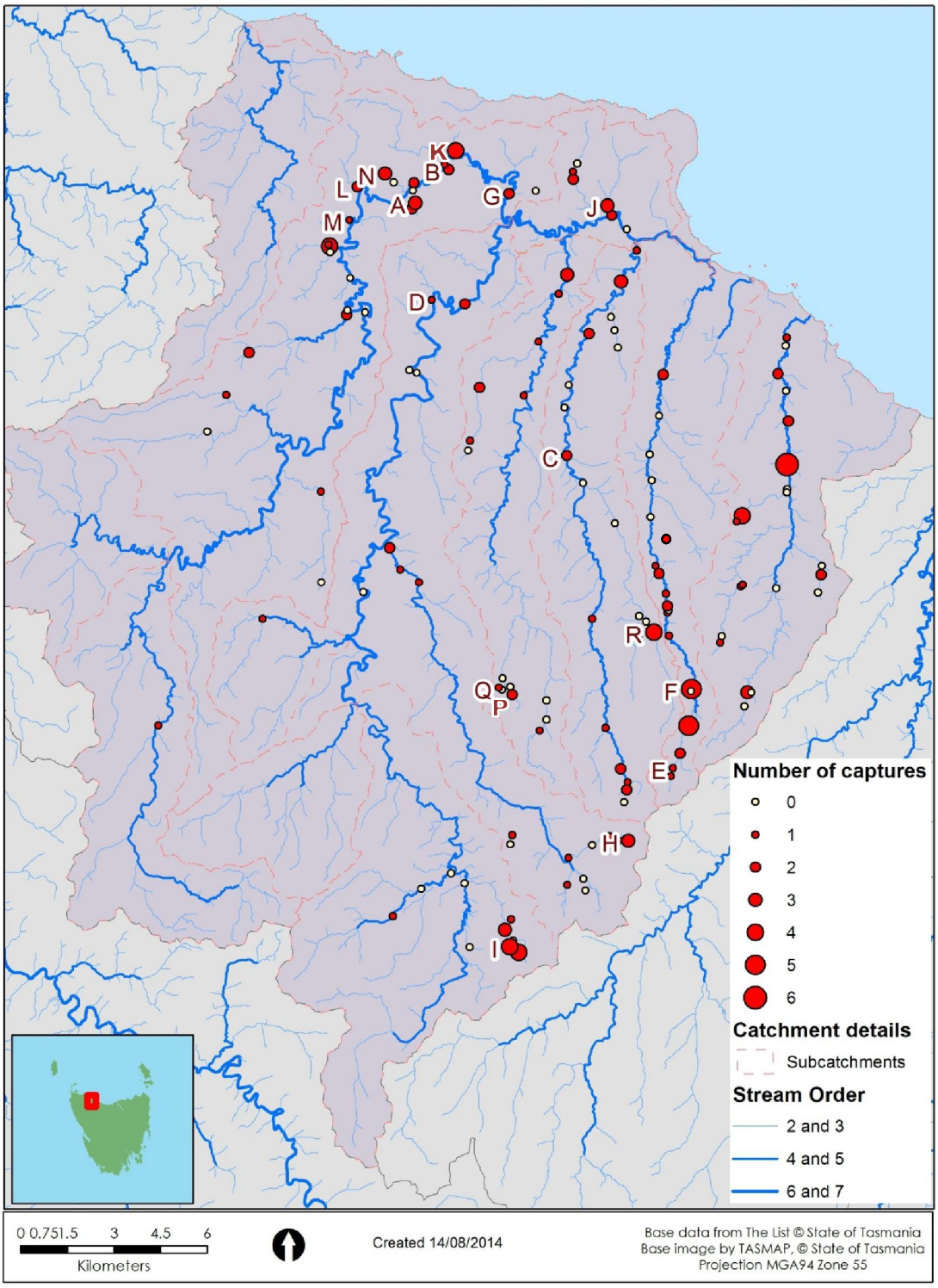


Table 2.3. Number of platypus captures in each subcatchment. The number of captures for each sub-catchment was not significantly different from the average capture rate across all sub-catchments (χ^2 test).

Subcatchment	Total number of captures	χ^2_1	p	Number of individuals			
				Adults	Sub-adults	Juveniles	Total
Lower Inglis River	11	0.44	0.505	9	1	0	10
Flowerdale River	33	0.53	0.468	31	1	1	33
Inglis River	15	0.54	0.464	13	1	1	15
Blackfish Creek	13	1.11	0.291	12	0	1	13
Big Creek	23	0.74	0.389	19	1	0	20
Camp Creek	27	0.68	0.411	20	2	2	24
Upper Inglis	13	0.00	0.964	12	0	1	13
Garners Creek	1	0.06	0.803	1	0	0	1
U. Flowerdale	1	0.03	0.873	1	0	0	1
Seabrook Creek	29	0.29	0.588	21	0	3	24

Table 2.4. Number of individual adult platypuses captured and sex ratios (female:male) for each subcatchment.

Subcatchment	Adult female	Adult males	Sex ratio
Lower Inglis River	6	3	2
Flowerdale River	14	17	0.82
Inglis River	7	6	1.17
Blackfish Creek	6	6	1.00
Big Creek	5	14	0.36
Camp Creek	8	12	0.67
Upper Inglis	6	6	1.00
Garners Creek	0	1	N/A
Upper Flowerdale	1	0	N/A
Seabrook Creek	10	11	0.91

A Shapiro-Wilk test did not show the distribution of the subcatchment sex ratio data (Table 2.4) to significantly differ from a normal distribution (p=0.223) and no outlier or extreme values were identified by a box and whisker plot. Although two thirds (67%) of the captures in spring were males and females made up 58% of captures in winter (Table 2.5), the sex ratio of platypus captures did not vary significantly between seasons (χ^2_1=4.47, p=0.22).

2.3.2 Time of year of captures

Platypuses were captured in all seasons and months as shown in Tables 2.5 and 2.6, and Figure 2.13. Capture numbers based on expected values for capture effort varied significantly across the seasons (χ^2_3=14.52, p=0.002) and there were significantly more captures by capture effort in spring than in other seasons (χ^2_1=8.88, p=0.003). However, actual numbers of platypuses captured in each subcatchment and overall did not vary significantly between seasons (see Table 2.5 for statistics).

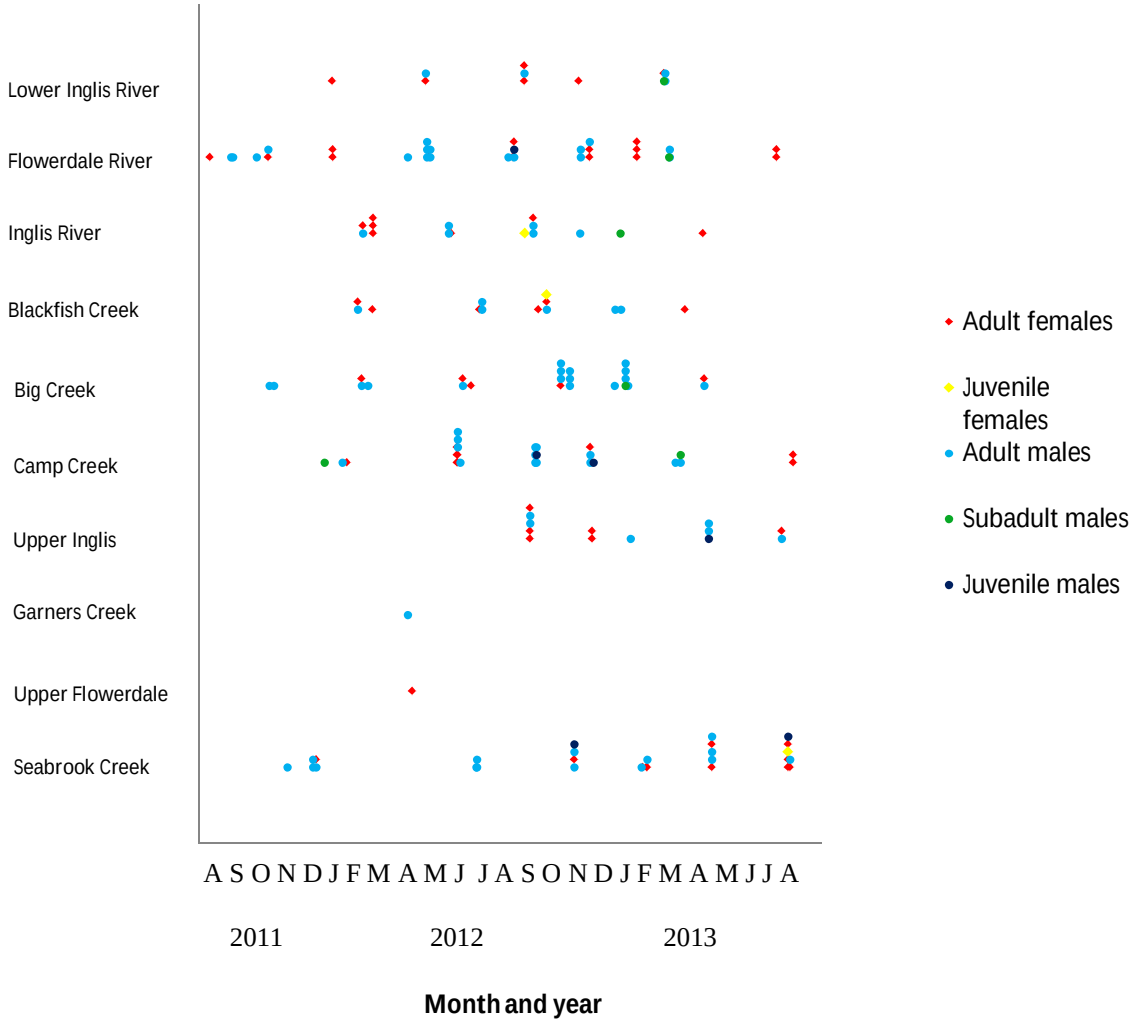
Table 2.5. Number of individuals captured in each subcatchment in each season.
* One individual captured twice in this season but only counted once in this table.

Subcatchment	Number of individuals captured in spring. Total (adult female:adult male)	Number of individuals captured in summer. Total (adult female:adult male)	Number of individuals captured in autumn. Total (adult female:adult male)	Number of individuals captured in winter. Total (adult female:adult male)	χ^2_1	p
Lower Inglis River	3 (2:1)	2 (2:0)	6 (2:3)	0 (N/A)	6.82	0.078
Flowerdale River	10 (3:6)	10 (7:3)	10 (1:8)	3 (3:0)	4.45	0.216
Inglis River	4 (1:2)	2 (0:1)	6 (5:1)	3 (1:2)	2.33	0.506
Blackfish Creek	4 (2:1)	2 (0:2)	4 (3:1)	3 (1:2)	0.85	0.838
Big Creek	8* (1:7)	6 (0:5)	4* (2:2)	3 (2:1)	2.81	0.422
Camp Creek	6 (0:5)	7 (2:3)	3 (0:2)	11 (7:4)	4.85	0.183
Upper Inglis	5 (3:2)	3 (2:1)	3 (0:2)	2 (1:1)	1.46	0.691
Garners Creek	0 (N/A)	0 (N/A)	1 (0:1)	0 (N/A)	3	0.392
U. Flowerdale	0 (N/A)	0 (N/A)	1 (1:0)	0 (N/A)	3	0.392
Seabrook Creek	4 (1:2)	9* (4:5)	5 (2:3)	10 (4:4)	3.71	0.294
OVERALL	**44 (13:26)**	**41 (17:20)**	**43 (16:23)**	**35 (19:14)**	**1.2**	**0.754**

Table 2.6. Number of juvenile and subadult platypuses captured in each season.

	Spring	Summer	Autumn	Winter
Juvenile	5	1	1	2
Subadult	N/A	3	3	N/A

Figure 2.13. Platypus captures by sex, age, date and subcatchment.



2.3.3 Capture rates (time after sunset, location and season)

Nets were in place at individual sites for 416 hours before sunset and 1135 hours after sunset. Only three of the 166 platypus captures were before sunset, which was lower than predicted if there had been an equal probability of captures across all net hours (χ^2_1=54.71, p=0.001). Platypuses were captured at a rate that largely reflected capture effort, although capture rate was lower for the first hour after sunset than predicted from trapping effort and greater than predicted 4-5 hours after sunset (see statistics in Table 2.7). The capture rates after sunset for the 10 subcatchments are shown in Table 2.8.

Table 2.7. Capture effort and number of platypuses captured for each hour after sunset. Bold values indicate significantly lower (↓) or higher (↑) trapping rates than predicted calculated assuming an equal probability of capture for each trap hour.

Time after sunset	Capture effort (hours)	Number of platypuses captured.	Number of captures per net pair hour.	χ^2_1	p
<1 hour	277.73	25	0.09	↓**8.06**	**0.005**
1-2 hours	261.30	41	0.16	0.31	0.577
2-3 hours	231.22	40	0.17	1.37	0.242
3-4 hours	172.17	23	0.13	0.13	0.723
4-5 hours	115.88	27	0.23	↑**6.41**	**0.011**
5-6 hours	55.92	7	0.13	0.14	0.713
6-7 hours	15.78	3	0.19	0.24	0.628
7-8 hours	2.90	0	0.00	0.42	0.518
8-9 hours	0.38	0	0.00	0.05	0.815

Table 2.8. Capture rate (platypuses per net pair hour after sunset) by subcatchment and season: Total platypuses (adult platypuses).

Subcatchment	Spring	Summer	Autumn	Winter	**TOTAL**
Lower Inglis River	0.21 (0.21)	0.28 (0.28)	0.16 (0.13)	N/A	**0.18** (0.17)
Flowerdale River	0.17 (0.16)	0.25 (0.25)	0.13 (0.12)	0.07 (0.07)	**0.15** (0.14)
Inglis River	0.18 (0.14)	0.16 (0.08)	0.07 (0.07)	0.17 (0.17)	**0.11** (0.10)
Blackfish Creek	0.17 (0.13)	0.11 (0.11)	0.10 (0.10)	0.06 (0.06)	**0.10** (0.09)
Big Creek	0.22 (0.22)	0.18 (0.15)	0.10 (0.10)	0.06 (0.06)	**0.13** (0.12)
Camp Creek	0.36 (0.30)	0.17 (0.12)	0.09 (0.06)	0.19 (0.19)	**0.18** (0.15)
Upper Inglis	0.29 (0.29)	0.15 (0.15)	0.08 (0.05)	0.10 (0.10)	**0.14** (0.13)
Garners Creek	N/A	0.00 (0.00)	0.18 (0.18)	N/A	**0.11** (0.11)
Upper Flowerdale	N/A	0.00 (0.00)	0.20 (0.20)	N/A	**0.12** (0.12)
Seabrook Creek	1.08 (0.81)	0.16 (0.16)	0.31 (0.31)	0.13 (0.11)	**0.18** (0.16)
TOTAL	**0.23** (0.20)	**0.17** (0.15)	**0.12** (0.11)	**0.11** (0.11)	**0.15** (0.16)

Mixed-model ANOVA including fieldwork site and session number for that site as random factors did not show significant effects for either factor (session number $F_{3,132}$ = 1.82, p=0.147; fieldwork site $F_{137,139}$ = 1.00, p=0.501). However, although there was considerable overlap in values as would be expected with the low but highly variable capture rates frequently encountered in platypus fieldwork (Grant, 2012), and although not representing events at individual sites, Figures 2.14 and 2.15 show trends of decreasing capture numbers and capture rates with increasing session number in the overall data.

Figure 2.14. Mean number of new individuals captured by session number at each site. Error bars = positive standard deviation.

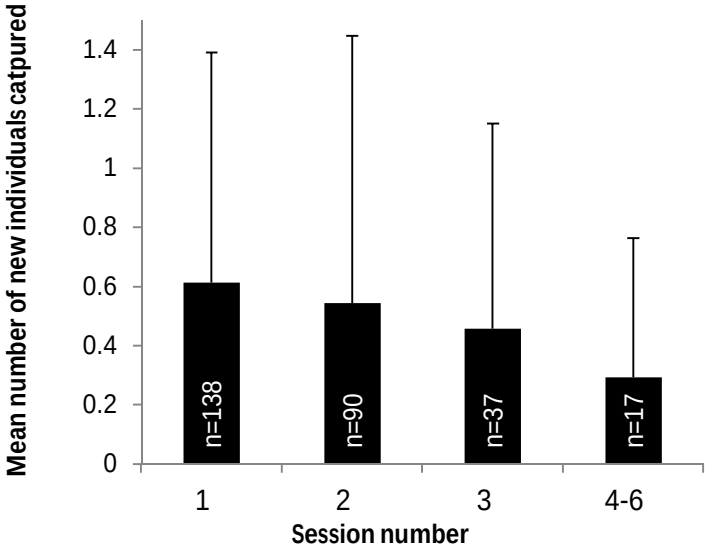
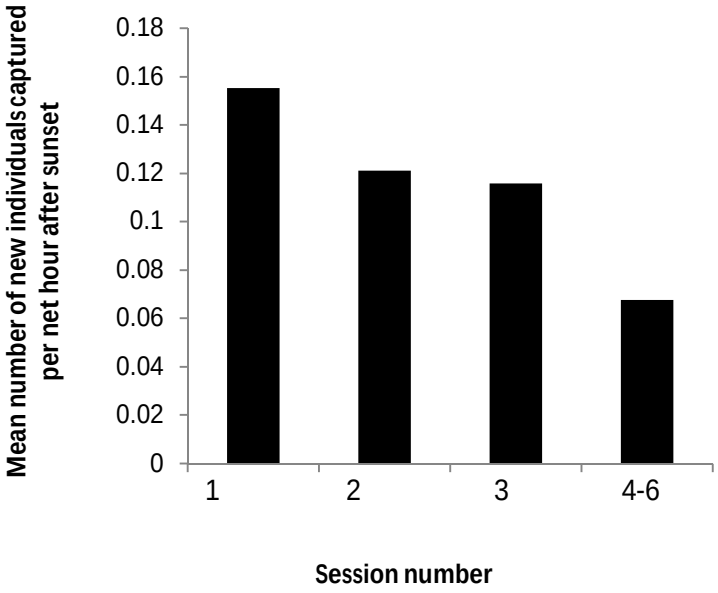


Figure 2.15. Mean capture rate of new individuals by session number at each site.



2.3.4 Survey of public sightings

The locations and frequencies of public sightings of platypuses in the area before 2012 are shown in Figure 2.16 and those during 2012 are shown in Figure 2.17. Of 101 locations where sightings were reported, four were in second order streams, one was in a third order stream, ten were in fourth order streams, eight were in sixth order streams, five were in seventh order streams and 73 were in farm dams. Six sighting locations were in tide affected stretches of river. Figure 2.18 illustrates the changes in frequencies at those sites for which data was gathered from the same person during the two time periods. This shows that an increase in frequency of sightings in 2012 when compared to previous years was only noted at one site. At 13 sites, there was a decrease in sighting frequency.

Figure 2.16. Location and frequency of public platypus sightings before 2012.

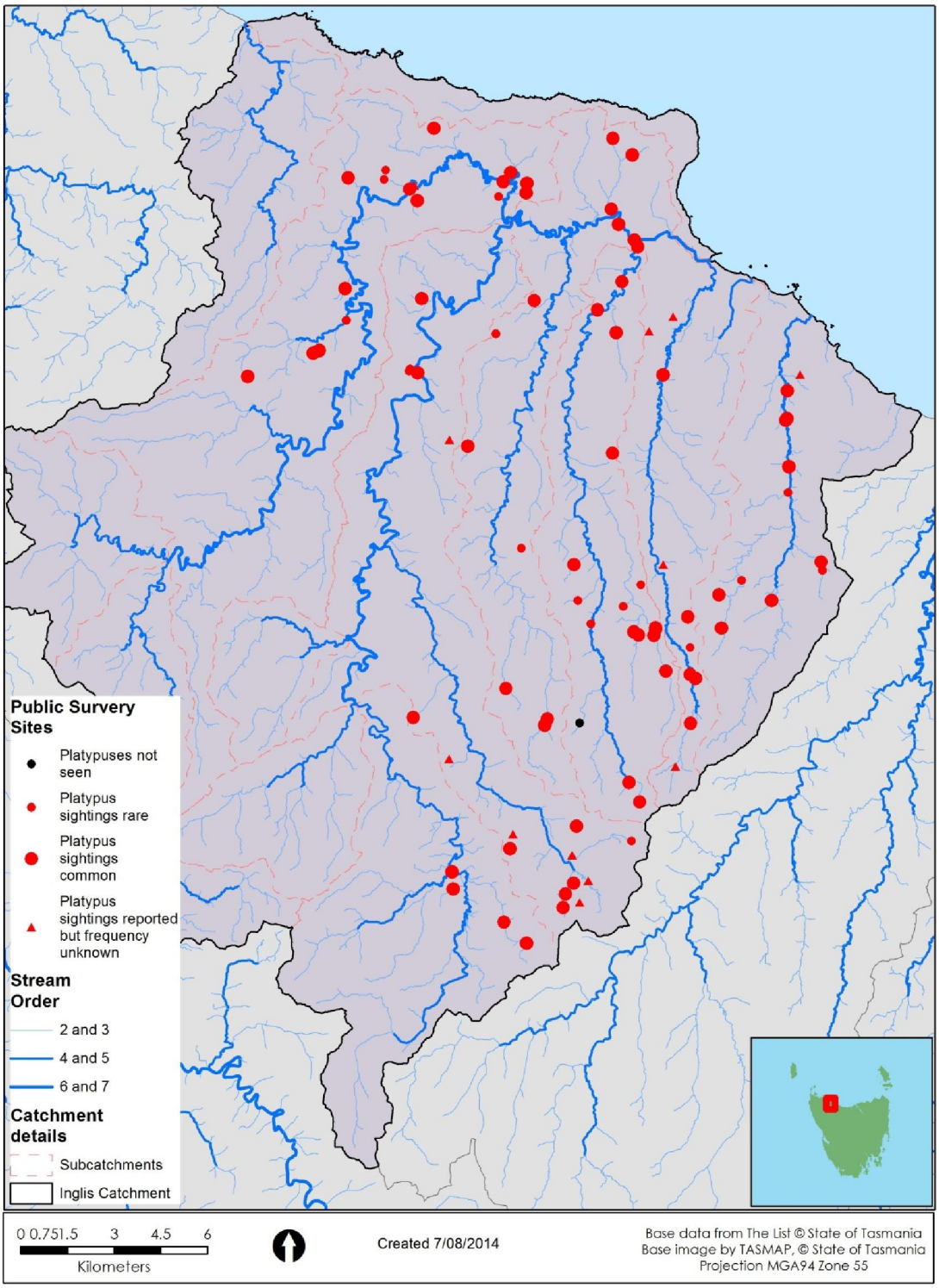


Figure 2.17. Location and frequency of public platypus sightings during 2012.

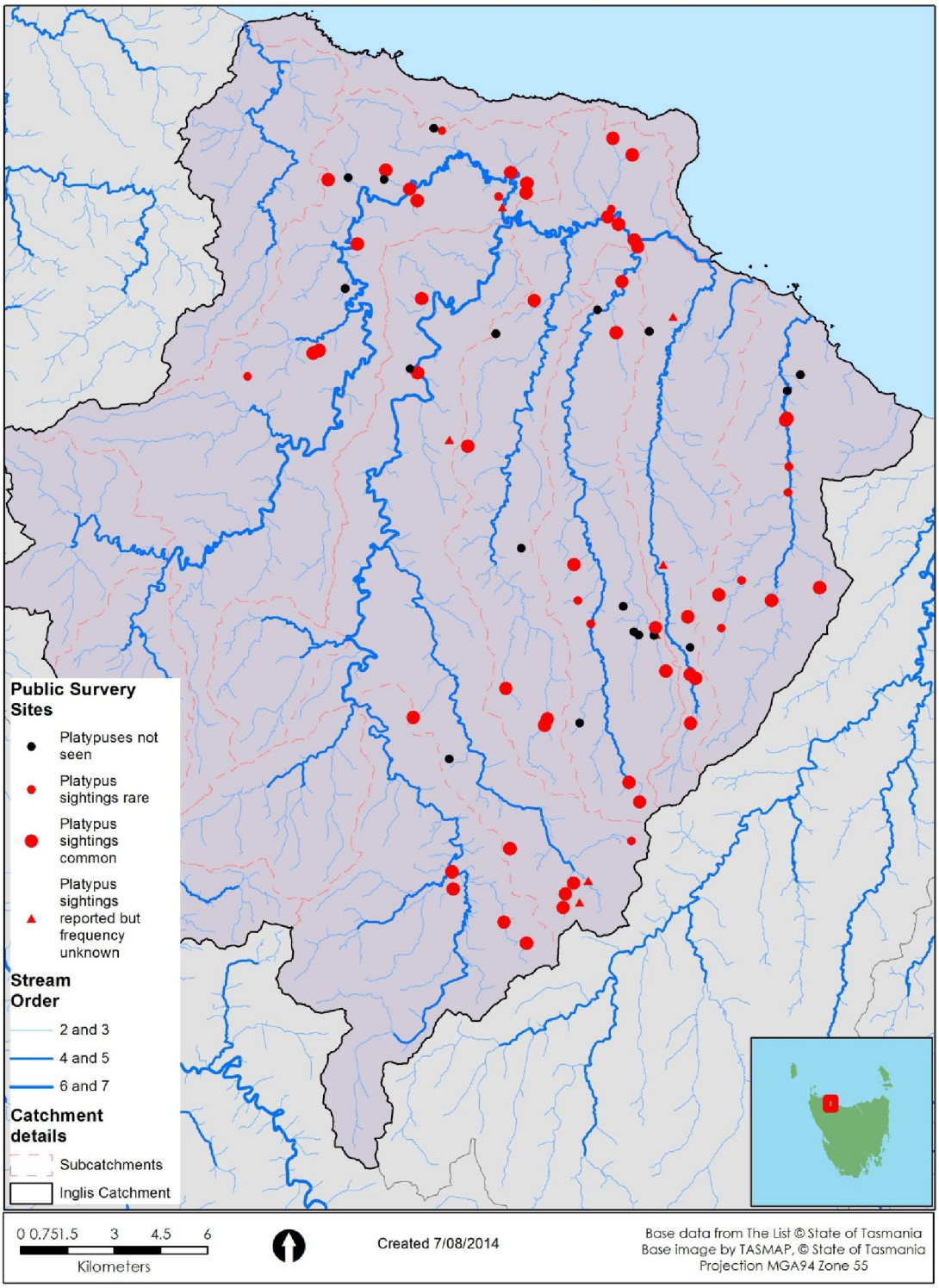
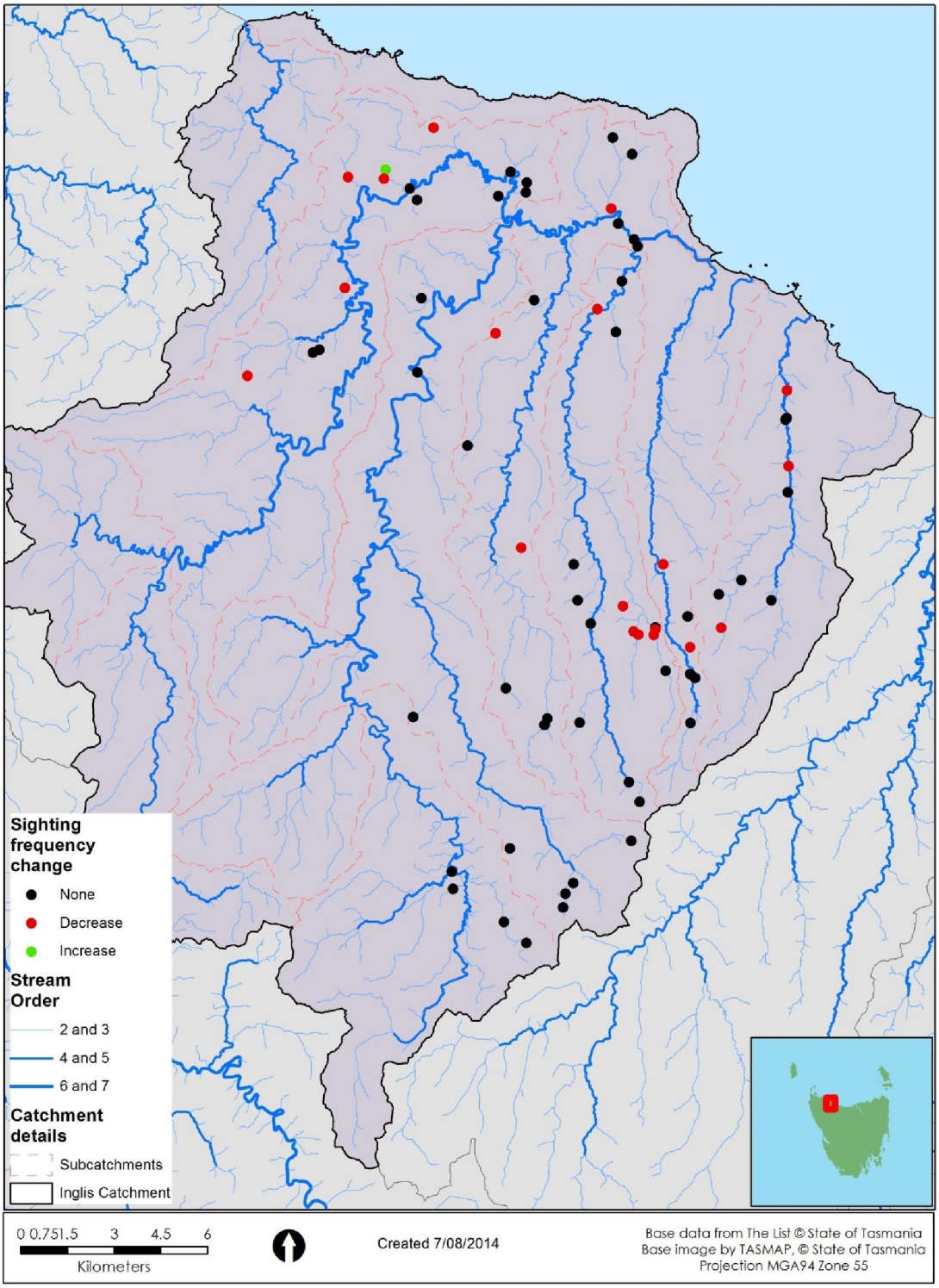


Figure 2.18. Relationship of frequency of public sightings in 2012 to that of sightings before 2012, for sites where data was available for both time periods from the same member of the public.



2.3.5 Habitat factors affecting capture numbers at each site

The results of forward stepwise regression examining the links between habitat factors and platypus capture numbers are shown in Table 2.9. Adult female captures were significantly, negatively correlated with amount of forest cover within a 500m radius. Total adult platypus captures were significantly, negatively correlated with amount of forest cover within a 500m radius, and non-significant correlations with the proportion of forested land in the relevant subcatchment, water area within 500m of connected water area, and altitude remained in the regression model.

Table 2.9. Results of forward stepwise regression of number of adult platypus captures during the first fieldwork session at each site against site habitat characteristics.

	Factors remaining in regression model	Beta	S.D. of Beta	B	S.D. of B	p level
Adult females	Forest cover	-0.214	0.091	0.000	0.000	**0.020**
	Subcatchment forest	0.105	0.091	0.322	0.278	0.249
Adult males	Forest cover	-0.135	0.086	0.000	0.000	0.118
All platypuses	Forest cover	-0.263	0.098	0.000	0.000	**0.009**
	Subcatchment forest	0.171	0.100	0.701	0.409	0.089
	Water area	0.111	0.092	0.000	0.000	0.228
	Altitude	-0.113	0.096	-0.001	0.001	0.244

2.3.6 Population size estimates

Following the methods described in Section 2.2.11 the calculations in Table 2.10 were used to estimate minimum observable population sizes of 600 for the Inglis River Catchment and 70 for the Seabrook creek catchment.

Table 2.10. Mimumum observable population size estimate calculations

		Inglis River Catchment	Seabrook Creek Catchment	Combined data from both catchments
A	Total surface area of water within 500m by water of the 138 capture sites (m^2)			1693067
B	Minimum number of platypuses using the fieldwork sites during the course of the project (including those captured in 2007-2008)			164
C	Minimum number of platypuses using the fieldwork sites on one night (ie adjusted for transient individuals) = B – (capture numbers x 0.09)			150
D	Mean number of platypuses per 10,000 m^2 of water within 500 m = (C/A)*10,000			0.886
E	Total area of dams in catchment (m^2)	4076190	633800	
F	Total area of rivers in catchment (m^2)	2553450	152650	
G	Total area of water in catchment (m^2) = E+F	6629640	786450	
H	Minimum number of observable platypuses in catchment (to one significant figure) = D*G	600	70	

2.4 DISCUSSION

This study provides baseline information on the distribution, age structure and sex ratio of the platypus populations in the Inglis Catchment. Such factors are vital to population health and are hence an important first step in the development of an assessment framework. An understanding of the environmental factors which may influence the occurrence of platypuses within the catchments was also gained. Both the field study and the public survey demonstrated a broad distribution of platypuses within the study area. In the field study, few individuals were captured in daylight hours and significantly more were captured in spring than in other seasons, based on the amount of capture effort. The proportion of captured platypuses that were juveniles was lower than

in other studies of similar or larger numbers of individuals, and more adult males than adult females were captured. A significant negative correlation was found between the number of platypuses captured at a particular site and the amount of forest cover (native, forestry or both) in the surrounding terrain. Studies with such a broad coverage of geography, habitat types and time within large platypus populations are scarce, and the approach of the study allowed it to be one of only a few to have attempted to estimate the size of a platypus population.

Table 2.5 demonstrates that, despite the unpredictability of platypus capture rates (Grant, 2012), the fieldwork largely achieved the goal of an even distribution of capture numbers between the four seasons overall and in each subcatchment. Figure 2.12 demonstrates the wide distribution of fieldwork sites and platypus captures within the two study populations. The low number of fieldwork sites in the southwest of the Inglis catchment is explained by the lack of vehicular access to most of this area. Compared to the other seasons, capture rates were significantly higher in spring, which includes the breeding season and early lactation (Chapter 5). This finding shows similarities to the findings of Serena and Williams (2012) who reported mean monthly capture rates for male (n =741 captures) and female (n = 585 captures) platypuses in Victoria over 15 years. Although no related statistical analyses were reported, the highest mean monthly capture rates for adult/subadult males occurred in July - September, which includes the early to middle parts of the breeding season in the study populations (Serena and Williams, 2012). There was less of a pattern for adult/subadult females, but the highest mean monthly capture rate occurred in January, during peak lactation.

Consistent with the findings of previous studies, platypus sightings reported in the public survey were widespread (Connolly and Obendorf, 1998; Otley, 2001); their distribution being similar to that of the platypus captures during the field study (Lunney *et al.*, 2004). The distribution of water bodies that respondents frequented was similar to the distribution of fieldwork sites, implying that as with fieldwork site selection, a lack of access to water bodies also limited the information gathered in the public survey. Public sightings were most common in farm dams and larger rivers. Sightings were reported from only five sites in rivers of Strahler stream order of three or less (Strahler, 1957). This likely relates to the area of water that can be observed at any particular time in the different water body types.

Only one location with no platypus sightings before 2012 was reported. Eighteen locations with no sightings in 2012 were reported. This difference is likely explained by the different lengths of the two time periods. For 19 locations, the reported frequency of observations was less in 2012 than for years before 2012. Since at 13 of these locations no platypuses observations were reported in 2012, the differing time period length may also have had an effect on this result. Rohweder and Baverstock (1999) attempted to compare current and historical sightings by asking respondents to document sightings in the previous five years and sightings prior to that five year period. This approach might reduce the effects of different time period lengths. However, accuracy of recollection even of recent events is an issue in any public survey (Bradburn *et al.*, 1987; Tourangeau, 2000), and it seems possible that it could be difficult for respondents to accurately determine the date of sightings occurring a few years ago (Bachman and O'Malley, 1981). While not quantified in this study, comments made at the end of the survey questionnaire implied that respondents had difficulty distinguishing between

frequency of observations and number of observations, the latter of which might vary with varying frequency of visits to rivers/dams. However, despite these potential limitations, the similarity of distribution of reported sightings to capture distribution provides some validation of the use of a public survey on the study populations and the results of this section of the project provide an important baseline for future studies that may choose to investigate platypus distribution in this area without undertaking fieldwork.

The sex ratio of adults captured in each subcatchment varied from 2:1 to 0.36:1 (Table 2.4) but the distribution of values was not significantly different from a normal distribution. Although not all previous studies have been explicit about whether subadult males have been classed as adults or juveniles, all but the lowest and highest subcatchment values in this study lie within the range of previously reported values (Grant, 2004; Gust and Griffiths, 2011). However, given the relatively low numbers captured in each subcatchment, further research would be required to determine if this is a consistent finding. It is important to note that the method of determination can introduce bias into sex ratio values (Mayr, 1939). Grant (2004), who reported a female:male sex ratio of 1.65:1 from a total 469 adults captured over 30 years used gill nets to capture platypuses; whereas Serena and Williams (2012) who reported a sex ratio of 0.79:1 from 1326 adult/subadults captures/recaptures, and Gust and Griffiths (2011) who reported a sex ratio of 0.66:1 in 166 platypuses and this study that observed an overall sex ratio of 0.83:1 in 139 adults all used fyke nets to capture platypuses. Gill nets are set in deeper, still or slow moving water and fyke nets are set in shallower slow or fast moving water. It is possible that differences in habitat use by the sexes (also suggested by the remote monitoring section of this project – see Section 3.4) combined

with differing capture methods may in part be the cause of observed differences in sex bias between field studies.

Of the total number of individuals captured in this study, 2% were juvenile females, 4% were juvenile male and 4% were subadult males. As with adult sex ratios, an issue arises in comparisons of juvenile capture proportions with those in previous studies as a result of some studies not stating whether subadult males were treated as adults or juveniles. However, juvenile capture proportions of 33%, 24% and 15% have been reported (Grant, 2004; Gust and Griffiths, 2011; Serena and Williams, 2012). Gust and Griffiths (2011) also noted that the proportion of juvenile platypuses in catchments that were affected by the fungal disease mucormycosis at the time was at least twice as high as those in other catchments. The lower figures observed in this study may represent a greater adult survivorship within the populations, lower birth rates, lower survival rates of juveniles/subadults or bias introduced by habitat use and capture methods.

Only three (2%) of 166 captures occurred during the 27% of overall net hours that were before sunset. This significantly lower figure is consistent with the findings of Serena and Williams (2012) and Koch *et al.* (2006) who, respectively, reported that 0.2% and 5% of captures occurred in daytime. During the remote monitoring section of this project 604 (28.5%) of 2,117 platypus observations occurred in the day at one site, and at the 15 other sites combined, 116 (7.7%) of 1505 platypus observations occurred in the day (see Section 3.3). Platypuses are therefore clearly active during the day, but are rarely being captured. The disturbance caused by setting the nets could reduce daytime capture rates. However, the average time a pair of nets was in place before sunset was 1.5 hours and for 32% of net pairs was >2 hours, which should be sufficient time to

have reduced effect of net-setting disturbance. As suggested by Serena and Williams (2012), a more likely reason for the low capture rate during daylight hours is that platypuses can see and therefore avoid the nets during daylight. Consequently, the use of net hours after sunset as the main unit of capture effort seems appropriate and is consistent with the approach of Serena and Williams (2012). Capture rates in this study were high compared to those in previous studies, which was likely to have at least in part been a result of performing fieldwork during the hours of greatest platypus activity (Figure 3.8). Platypus recapture rates, even in studies performed over decades, are generally low (Bethge, 2002; Serena and Williams, 2012). Avoidance of fyke nets by a previously captured platypus has been demonstrated (Griffiths *et al.*, 2013). The low recapture rates in this study are consistent with these findings. Few recaptures at the site of initial capture for the >80% of platypuses that appeared to be resident in one area (see Section 3.3) would be expected due to the low number of sessions at each site (average 2.03). Few recaptures of transient individuals at a site other than that of initial capture would be expected due to the wide geographical distribution of the fieldwork sites (Figure 2.3).

Investigation into the effects of broad habitat characteristics on platypus capture numbers at each site indicated a negative correlation between the amount of forest within a 500m radius of the fieldwork site and the number of captures. Two possible explanations for these results are: 1) that removal of forest for agriculture in certain areas has been associated with a local increase in platypus population density, or 2) that deforestation, largely for agriculture, has occurred in those areas that are naturally more productive, naturally supporting a larger benthic macroinvertebrate community and

hence a greater platypus population density. The results of this and previous studies suggests that the latter explanation is more likely to be correct.

Kruuk (1993) suggested that although eutrophication - increased nutrient concentrations common in areas impacted by human activities - may reduce the diversity of macroinvertebrates in waterbodies, it may increase the overall density of macroinvertebrates allowing increased foraging efficiency for platypuses. This process would be consistent with an association between deforestation and an increase in local platypus population density. However, if lower amounts of local forest area (i.e. within 500m) were correlated with higher platypus population densities due to local eutrophication and increased food availability, the observed positive relationship between the amount of forest area at a larger special scale (i.e. subcatchment forest area) and capture numbers, although not statistically significant, would not be expected to remain in the regression models for female and total platypuses.

In support of this, the findings of two previous reports suggest that eutrophication has not had a major impact of benthic macroinvertebrate communities in the study populations. Bobbi *et al.* (2003) reported the findings of water quality assessments at 27 sites in the Inglis River and Seabrook Creek Catchments from 1999 to 2001, while Warfe (2003) reported the findings of investigations into benthic macroinvertebrate communities at 40 sites in the Inglis River and Seabrook Creek Catchments in 1999. Bobbi *et al.* (2003) observed elevated nitrate concentrations, more seasonally variable nitrate concentrations and elevated phosphorus concentrations, all consistent with a degree of eutrophication, in agriculture dominated areas of the Inglis River and Seabrook Creek Catchments. However, although Warfe (2003) did not report

quantitative results for benthic macroinvertebrate communities, the qualitative results were considered in general not to be consistent with the effects of eutrophication. Reduction of benthic macroinvertebrate diversity was reported at eight sites, but at all but two sites did not consist of a loss of nutrient sensitive groups as would be expected if eutrophication were the cause of the reduced macroinvertebrate diversity. Increased sedimentation - from land clearing, riparian vegetation removal and modification, stock access to rivers, forestry operations, gravel roads and gravel pits - was considered to be the cause (Warfe, 2003).

In light of these findings, it seems more likely that the higher platypus numbers in the areas dominated by agriculture are because these parts of the broader catchment are naturally more productive and favourable for both the platypus and agricultural purposes. While further research is required to investigate this suggestion, it could have implications for the selection of sites for conservation areas. It may be that to take into account the natural distribution of certain species, including the platypus, conservation areas should include naturally more productive areas as well as those that are unsuitable for agriculture +/- forestry. Lunney *et al.* (2004) observed that while platypuses still occur in modified habitats, this may not continue to be the case, and rehabilitation of disturbed riparian habitats and prevention of further degradation are required to ensure the conservation of the species.

Two other non-significant correlations remained in the regression model for total captures which may also shed light on platypus habitat preferences. Firstly, the positive correlation of total captures with water area within 500 m by connected waterway is consistent with the higher catch success of Koch *et al.* (2006) in higher order streams

and in farm dams, and could be explained by a relationship between food availability and the area of benthic macroinvertebrate habitat. This will be expanded upon in discussion of the minimum observable population estimates. Secondly, the negative correlation between total capture numbers and altitude could be explained by altitude related variation in climate and/or waterway productivity.

The difficulties of making estimates of platypus population size have been discussed (Gust and Griffiths, 2010; Grant, 2012) and only rarely have such estimates been made (Grant and Carrick, 1978; Serena, 1994; Fox *et al.*, 2004). However, given the reliance on estimating past or future population declines in the threatened species list assessment processes, difficulties associated with estimating platypus population size may result in errors when assessing the conservation status of the platypus (Grant, 2012).

This current project provided capture data from many locations distributed broadly within river catchments. Such data can be used to produce an estimate of population size. Field *et al.* (2007) suggested that wildlife population monitoring in Australia has generally lacked rigour, and encouraged the use of advanced techniques in quantitative ecology. However, when using a relatively complex metapopulation patch structure model, Fox *et al.* (2004) noted the limitations on such an approach presented by a lack of information regarding platypus biology, and also the sensitivity of the model to small changes in input data. The approach taken in this study, therefore, was to use the available data to produce relative population size index estimates using a process that is easily understood and that has easily identifiable limitations so that comparisons could more easily be made by future researchers.

The limitations of the minimum population estimates in this part of the project relate to assumptions made in the estimation process, as well as the fact that they are not estimates of actual population size. To determine the actual population size for a river catchment at any particular point in time all the platypuses in that river catchment would need to be counted at that time. The approach in this project varies from this gold standard approach in a number of ways. We investigated the number of platypuses captured at 138 sites across two river catchments, made assumptions about the linear distance along a waterway that platypuses swim in a night and extrapolated the number we might capture throughout the catchments (if unlimited time and resources were available) using the mean number of platypuses per unit of water surface within the nightly linear swimming distances of the capture sites as a measure of platypus density.

This approach has a number of clear limitations. Firstly, assumption 3 in Section 2.2.11 may be incorrect – i.e. the 138 field sites may not have been representative of the two study catchments. However sites were distributed as widely as possible by subcatchment, altitude and surrounding land use. Secondly, although water surface area remained in the regression models for the number of male captures and overall captures, these were not significant results. The figure for the overall number of platypuses per unit of surface area was based on an estimated mean linear distance along a waterbody that platypuses swim in a 24 hr period. The mean nightly swimming distance of 1000 m was chosen on the basis of the results of previous live capture and radiotracking studies (Carrick and Hughes, 1978; Serena, 1994; Gardner and Serena, 1995; Gust and Handasyde, 1995; Serena *et al.*, 1998; Serena and Williams, 2013). However, it is a) unlikely that this figure is exactly correct, and b) likely that nightly swimming distances are determined by a complex set of parameters and a mean value does not well represent

the movements of all the platypuses in the catchments (i.e. assumption 1 in Section 2.2.11) is likely to be incorrect. Equally, assumption 2) is likely to be incorrect – i.e. the relationship of the number of platypuses to local surface area is likely to vary between and within water bodies, and the number of platypuses is likely not to be related to local water surface area only. The number of platypuses per unit of water surface area may vary to some degree, amongst other things, according to the depth of the water, the distance from the bank and access to burrow sites. However, nearly all of the farm dams in the study areas are relatively narrow (<100 m) and shallow (<10 m; i.e. mostly within the usual the diving range of a platypus; Bethge et al., 2003). In Table 2.9, the beta value for the correlation between local water surface area and platypus capture numbers is low and other habitat variables remained in the regression models. However, inclusion of these factors (forest area within 500 m, subcatchment forest area, and altitude) in a population size index would require a complex model which, as described above, this project aimed to avoid. A relationship between water surface area and number of platypuses captured is supported to some extent by the higher catch success in higher order streams with a controlled capture effort per site by Koch *et al.* (2006), and the suggestion by Serena (1994) that capture numbers in a stream approximately 3m wide were increased by the presence of adjacent pond systems Similarly, Turnbull (1998) reported few platypus sightings in headwater streams in a public survey. The assumption by Fox *et al.* (2004) that, before adjustments (reductions) were made for low rainfall, plantation establishment, native forest harvesting and agriculture, the number of platypuses/km increased through stream order categories 1, 2-4 and 5-6 was also consistent with this aspect of this project's methodology.

Results of this and previous studies indicate that this current project may have captured approximately 30% (≈ 50%*{100-37%}; see below) of the non-net avoiding platypuses using each site on any particular day. The sources of this likely underestimation, discussed in more detail below, are as follows:

- Proportion of platypuses not captured due to low number of sessions at each site: 50%
- Proportion of platypuses not captured because they were only active in second half of night (30%), or the day (7%): 37%

Figure 2.14 indicates that if six sessions were performed at each site, on average a total of approximately 2.5 platypuses would be captured at each site. Given that Figure 3.6 indicates that ~80% of individuals continued to be present at the site of their capture during the course of the project, on average ~0.5 of this expected 2.5 platypuses per site would be transient individuals. As these captures of transient individuals would be expected to be spread evenly between fieldwork sessions, they would be expected to occur at an average rate of approximately 0.08 platypuses per session. Following on from this, it would appear that the values for session 4-6 had not yet reached figures expected if they consisted only of transient individuals or replacement of resident individuals. Reducing total captures by the expected number of transient platypuses captured at all but one session as above, the expected number of individual platypuses per site would be ~2.08 in six sessions. The average number of sessions per site was 2.03. Figure 2.14 indicates that on average ~1.06 individuals (excluding transient individuals from one session) were captured in the first two sessions at each site. This suggests that on average ~50% of the individuals expected to be captured during six

fieldwork sessions at each site were not captured. It is also possible that a small number of new resident individuals would be captured after the sixth session at each site.

Serena and Williams (2012) observed that, using fyke nets in Victoria, 37% of 1,326 adult/subadult captures and 27% of 316 juvenile captures occurred after 1am. However, results from the remote monitoring section of this project indicate that for most platypuses activity at a particular site occurs in both the first and second half of the night either on different nights, or sometimes on the same night. Hence multiple sessions in the first half of the night at the same site is likely to capture most of those platypuses that can be captured at that site. In this study, the nets were usually removed from the water between 11pm and midnight. The average number of sessions per site being 2.03, it is likely that more individuals could have been captured if nets were set until dawn or if more sessions were performed per site.

Bethge *et al.* (2009) observed strictly diurnal behaviour in 2 of 29 individuals in Tasmania, but these individuals were only monitored once for 7 days each. A third platypus showed diurnal behaviour during a monitoring period in winter, but not in summer. In a radiotracking study of male platypuses in Victoria, Gust and Handasyde (1995) observed that no individuals were active in the day in the non-breeding season, but in the breeding season, some individuals changed to a diurnal activity pattern. Figure 3.10 demonstrates that in this study, during an 18 month remote monitoring period, diurnal movement of each of four platypuses past a monitoring site was detected mostly during or shortly after the breeding season. Given that platypuses appear to rarely enter fyke nets in daytime (Serena and Williams, 2012), any strictly diurnal platypuses would not be captured using the methods of this project.

Griffiths has reported net avoidance in a single previously captured platypus and low recapture rates are common in platypus fieldwork (Grant, 2004; Serena and Williams, 2013). In this study, many platypuses that were regularly detected at the sites of their captures during the remote monitoring were not recaptured with repeated fieldwork sessions at those sites. However, the rate of net avoidance in previously uncaptured platypuses is unknown.

Bethge (2002) captured 52 individual platypuses over two years at Lake Lea (~142ha in size) in Tasmania. Of these, 28 were considered to be resident in the lake (captured at least twice over a period of greater than one year) and 24 were consider transient inhabitants of the lake. If it is assumed, arbitrarily, that six of these transient platypuses (and hence 34 platypuses in total) were living in or around Lake Lea at any point in time, the observable platypus population density was 0.24 platypuses/10,000 m^2. This figure is lower than the equivalent used in this project. It is possible that net avoidance by platypuses captured by Bethge (2002) led to underestimation of the number of resident individuals. It is also possible that net avoidance by platypuses captured by previous projects at the same location (Otley *et al.*, 2000), and a lack of fieldwork in certain parts of the lake (Sarah Munks, personal communication) led to an underestimate of both the total and the resident populations. However, the relatively low population density could relate to the habitat characteristics of this location. For instance, access to burrow sites may be a more important factor limiting population density in larger water bodies. The negative effect of altitude on capture numbers observed in this study might lead to a reduced number of platypuses at Lake Lea (870 m altitude) when compared to a similar area of water in the Inglis River and Seabrook

Creek Catchments (<590 m in altitude). Additionally, differential use of areas of the lake may affect platypus density. Although Lake Lea is mostly less than 2m deep and almost entirely less than maximum dive depth observed (Bethge, 2003), use of the edge of the lake appeared to be different to that of the centre, with platypuses spending more time foraging within 100-150 m of the shore (Bethge, 2002).

Grant and Carrick (1978) estimated platypus numbers in a 1.8 km section of the upper Shoalhaven River in NSW, consisting of a 940 m pool and a 500 m pool. Estimating the width of the river to be on average 20 m in the longer pool, 8 m for the shorter pool and 6 m for the riffle area (Tom Grant, personal communication), the total surface area of this stretch of river is estimated to have been ~25,000m^2. Grant and Carrick (1978) estimated that this section of river had a resident population of 14-18 platypuses, equating to ~5.6-7.2 platypuses/10,000 m^2 of water.

During three consecutive years, Serena (1994) observed adult/subadult platypus populations of 1.3-2.1 adult platypuses/km over different lengths of an 8 km section of river. Dimensions were given for the whole 8 km of river, not for the different parts studied in each of the three years, and a total water surface area of approximately 30,000m^2 in the study area can be calculated. If the capture numbers are extrapolated to the whole 8 km of river, a population density of 3.5-5.6 platypuses/10,000m^2 of water surface area can be estimated. However, if it had been possible to take into account individuals that only transiently inhabited the area, as well as habitat use outside of the study area, the observed population density may have been lower.

The similarities between the minimum population density estimate calculated in this study (0.9 platypuses per 10,000m^2) and those estimated from the results of three previous studies suggest that there is merit to the approach taken. Although some factors that, on the basis of regression models, appear to have affected platypus capture numbers at the site level (surrounding forest area, subcatchment forest area, and altitude), were not used in the minimum population estimates at the catchment level, confidence in the minimum population estimates is based in part on the broad distribution of the fieldwork sites across land-uses, subcatchments and altitudes within the two study catchments. Given the current paucity of available information on platypus habitat preferences, and the sensitivity of complex models to changes in input data (Fox *et al.*, 2004) it is hoped that the approach taken is not only open, but also is as reliable as possible. It is anticipated that continued platypus capture at the fieldwork sites in this project, and continued use of in-stream microchip readers (See Chapter 3) will provide minimum population estimates closer to the actual population sizes.

Chapter 3.

Remote monitoring

3.1 INTRODUCTION

Individual survivorship, longevity and use of habitat are important aspects of the natural ecology of wild species' populations as well as the impacts of the conservation threats they face. Research in these areas in platypuses has largely relied on recapture of individuals or application of tracking devices. This has provided useful information but both methods are resource intensive and have logistical limitations and few studies have explored alternative approaches.

Grant (2004) and Serena and Williams (2013) have both carried out long term capture-recapture studies that have shed light on the longevity of platypuses. In his capture-recapture study of 700 platypuses over 30 years in the upper Shoalhaven River, New South Wales, Grant (2004) observed two females first captured as juveniles 13 and 21 years previously and an additional seven juveniles captured again at between five and eight years of age. Grant (2004) also captured 32 five to 15 years after their initial capture as adults. One juvenile male was recaptured at two years of age and four were recaptured at one year of age. Of the males that were initially captured as adults, 32 were recaptured three to seven years later (Grant, 2004). Serena and Williams (2013) reported the findings of fieldwork performed in Melbourne, and in western Victoria between 1995 and 2007. In the Melbourne area, adult males and adult female platypuses were recaptured over periods of up to 117 (mean = 45.9±5.4) months and 127 (mean = 43.9±5.2) months, respectively (Serena and Williams, 2013). In western Victoria, adult males and adult female platypuses were recaptured over periods of up to 90 (mean = 31.9±8.6) months and 72 (mean = 19.0±5.8) months, respectively (Serena and Williams, 2013). Williams *et al.* (2013) reported known ages of up to and greater than 48 months

for platypuses initially captured as juveniles but did not report the maximum known age individuals (Williams *et al.*, 2013).

Platypus movements have been studied using radiotelemetry, dataloggers and, to a lesser extent, capture-recapture (summarized in Tables 3.1 and 3.2 and references therein). Radiotelemetry and dataloggers have allowed the patterns of den use and daily activity patterns to be observed and have revealed that platypuses generally enter and exit their burrow once a day (Serena, 1994). Estimates of activity times suggest that platypus activity bouts last between 3 – 30 h, possibly with longer activity periods during winter and spring (Table 3.1). Activity periods occur mostly during the night, but there is evidence of greater day time activity in the breeding season, in winter and also possibly in Tasmania. Male platypuses appear to have larger home ranges than females, and platypuses appear to have a core area within their home range that is used extensively (Table 3.2). Overlap of home ranges between platypuses of all age/sex categories appears to be common but there is evidence of spatial and temporal separation of individuals within the overlapping areas (Table 3.2). Extensive dispersal has been observed, but only in two juvenile males (Table 3.2).

Table 3.1. Observations of platypus (*Ornithorhynchus anatinus*) activity periods from radiotracking studies.

Reference	Location	Lentic/ lotic	Sex	Time active outside burrow		Findings		Other findings
				Mean (h/day)	Range (h/active period)	Diurnal patterns	Seasonal patterns	
Grant *et al.*, 1992	Thredbo and Shoalhaven Rivers, NSW	Lotic	Males and females	10.7±3.5	8.9-18.75	Primarily, but not exclusively, at night		
Gust and Handasyde (1995)		Lotic	Adult males	~ 10		Active mostly at night	40% of animals changed from nighttime to daytime activity during breeding season	
Serena (1994)	Badger Creek, Vic.	Lotic	Males and females, adult and non-adult	7.3-12.4	7-20.2	Typically active at night but daytime activity also recorded		
Otley et al. (2000)	Lake Lea, Tas.	Lentic	Adult males and females	8.5-15		50% active in day in winter. 20% active in day in summer	Possibly longer periods of activity in winter	
Bethge (2003)	Lake Lea, Tas.	Lentic	Males and females, adult and subadult	10.0-15.8	3.4-30.8	61% active in night, 8% active in day, 31% mixed.	Activity periods longer between August and November. Strictly daytime behaviour only in winter in 3 females	16% of female active periods were diurnal. 2% of male active periods were diurnal

Table 3.2. Home range estimates for platypus (*Ornithorhynchus anatinus*) from previous studies.

Reference	Location	Lentic/ lotic	Method	Sex	Period of estimate	Linear home range (m)	Home range area (ha)	Seasonal patterns	Overlapping Home ranges?	Other findings
Otley *et al.* (2000)	Lake Lea in northwest Tasmania	Lentic	Radiotracking	Adult males and females	24 hours		2-58		Yes	
Gust and Handasyde (1995)	Goulburn River, Vic.	Lotic	Radiotracking	Males	few weeks	350-2,575	2.45-15.45	No significant difference with breeding season or changing water level	Yes. Possible spatial separation and temporal separation within overlapping ranges	Intensive use of core 30.5% ± 9.3of home range
Serena, 1994	Badger Creek, Vic.	Lotic	Radiotracking	Males and females, adults and non-adults	Up to a few weeks	330-2,280			Yes, between several combinations of age/sex classes	24-70% of home range used in a given 24 hour period
Serena *et al.*, 1998	Yarra River and two tributaries, Vic	Lotic	Radiotracking	Males and females, adults and non-adults	5-76 days	2,900-7,300			Yes	
Grant *et al.*, 1992	Thredbo River, NSW	Lotic	Radiotracking	Males Females	2-9 days	400-2,300 400-600				
Gardner and Serena, 1995	Yarra River and three tributaries, Vic	Lotic	Radiotracking	Males	Several weeks	2,900-7,000			Yes, but males largely used different parts of shared areas	2.0±1.4km used per activity period
Serena and Williams (2013)	Streams and rivers near Melbourne, Vic.	Lotic	Capture-recapture	Males Females	25-117 mo 47-127 mo	6,000-13,900 Up to at least 4,400	3.0-6.9* Up to at least 2.2*			2 juvenile males moved >40 km

* Linear distances are converted to home range areas by approximating the width of the creeks in the study area to be 5 m.

Aside from the labour intensive nature of the methods used in live capture and radiotelemetry/datalogger studies, each has specific limitations in relation to the gathering of data. The main limitation of live capture as a method of monitoring platypus movements is that recapture rates are generally low (31-58%; Grant, 2004), Serena and Williams, 2013), and therefore obtaining data on movement patterns is both limited and extremely time consuming. Grant (2004) suggested that the low recapture rate of platypuses may in part be a result of greater long-term mobility over periods in some individuals. However, net avoidance as observed by Griffiths *et al.* (2013), may also be a reason for low recapture rates. The main limitation of radiotelemetry and dataloggers is that they can only be used for periods of up to several weeks due to battery life and the detachment of devices glued to platypuses' backs.

The use of acoustic transmitters to monitor platypus movements has been reported (Griffiths *et al.*, 2013). However, at the time of writing, the extent of the research reported was limited to net avoidance in one individual (Griffiths *et al.*, 2013). Macgregor et al. (2015_Appendix E) and Appendix F demonstrated the use of in-stream microchip readers to be an effective method of remotely monitoring platypus movements and survivorship in suitable streams. This method is relatively non-labour intensive and can be used for long-term monitoring. The approach relies only on routine identification of individuals with microchips with only a single capture of the animal required (to insert the microchip). The primary aim of this part of the project was to investigate platypus survivorship (to assist assessment of the effects of individual health, genomic and demographic observations in the population health assessment framework). A secondary aim was to provide insights into platypus movements, behaviour and migration in the study catchments.

3.2 METHODS

A field study was performed between November 2011 and May 2014, using in-stream microchip reader units to monitor the movements of wild platypuses (Macgregor *et al.*, 2015). Platypus movements were monitored at 18 specific sites (A-R) in the Inglis Catchment in northwest Tasmania (Figures 3.1, 3.2 and 3.3). Between sites A-H and J-R, a total of 37 monitoring periods were undertaken (mean 2.2 ± 1 per site), ranging in length from 8 to 69 days (mean = 26 ± 12 days). At Site I (Figure 3.3 and 3.4), monitoring occurred for a single continuous period of 548 days.

Figure 3.1. Locations of platypus capture and monitoring sites in the Inglis Catchment, Tasmania: a) red dots: animals identified with Trovan Unique® microchips (August 2011-December 2012), letters: sites monitored using in-stream antennae between November 2011 and December 2012; b) purple dots: animals identified with ISO microchips (December 2007-August 2008), letters: sites monitored using in-stream antennae capable of detecting ISO microchips between November 2011 and December 2012.

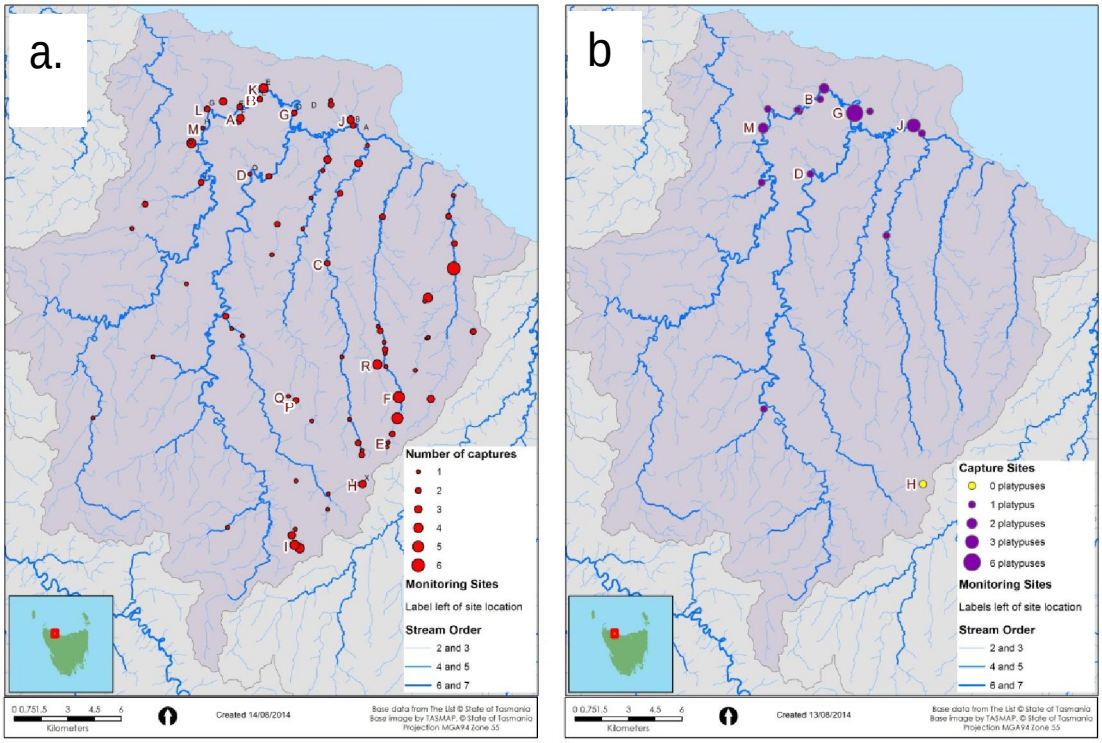


Figure 3.2. Monitoring sites A-H and K-H (labelled). Arrow indicates antenna position.

Figure 3.2 (Cont'd). Monitoring sites (labelled). Arrow indicates antenna position

Figure 3.2 (Cont'd). Monitoring sites (labelled). Arrow indicates antenna position

Figure 3.2 (Cont'd). Monitoring sites (labelled). Arrow indicates antenna position

Figure 3.2 (Cont'd). Monitoring sites (labelled). Arrow indicates antenna position

Figure 3.3. Site I, where monitoring occurred for a single continuous period of 548 days a) position of antenna [arrow] in creek, b) looking downstream to large farm dam in summer, c) looking upstream to small farm dam, and d) looking downstream in spring.

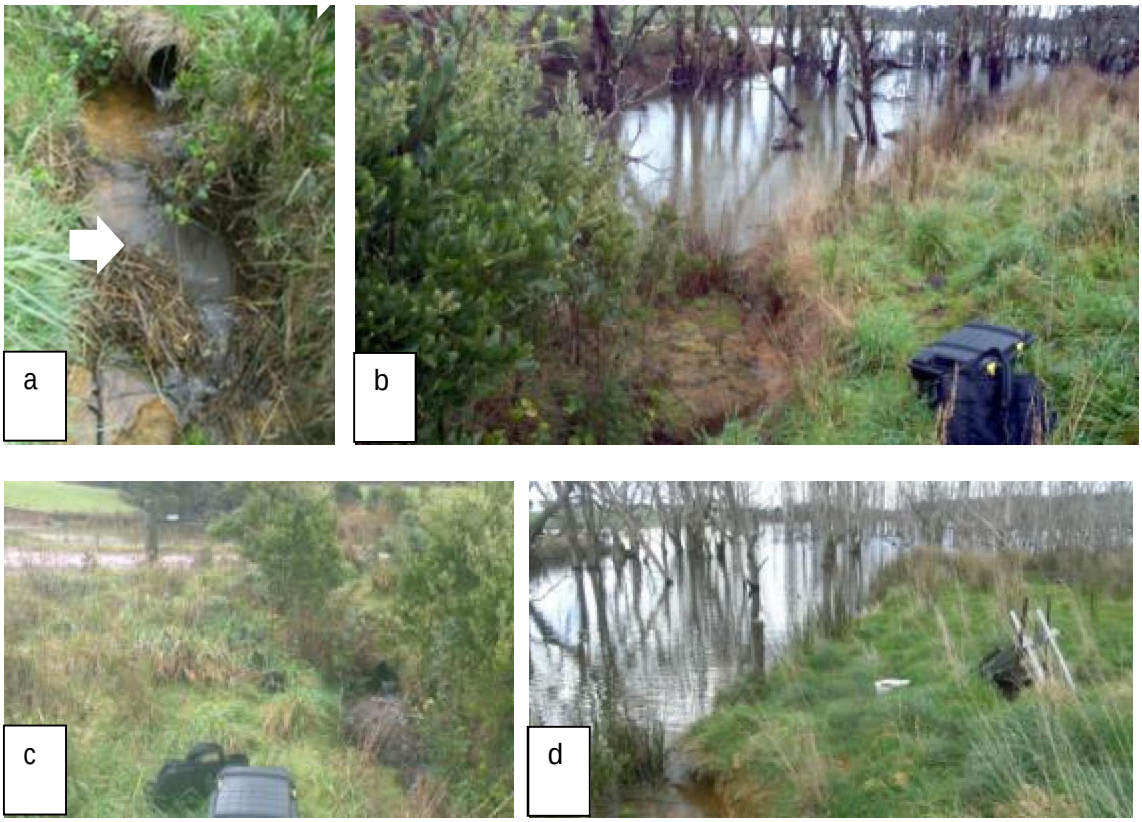
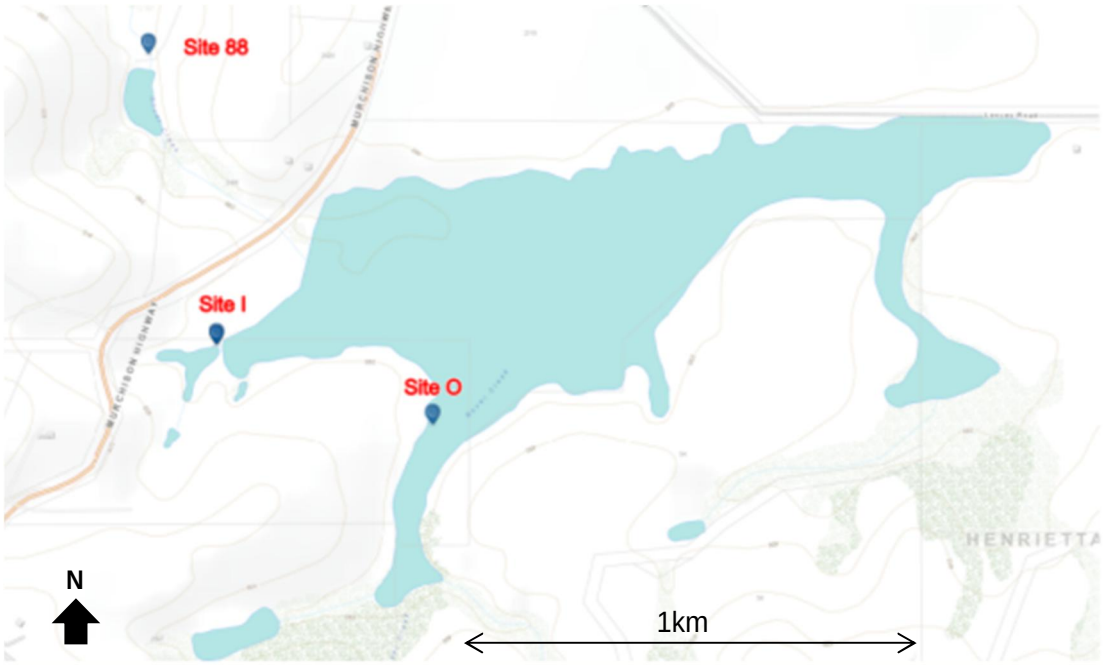


Figure 3.4. Location of Site I in detail (source http://maps.thelist.tas.gov.au**).**



A total of 31 platypuses had been microchipped in the Inglis Catchment before commencement of the in-stream microchip monitoring work: 23 (10 adult males, two juvenile males and 11 adult females) between December 2007 and August 2008 with ISO microchips (Reunite®, Novartis Animal Health Australia, North Ryde, NSW; Macgregor *et al.* 2010) and 8 (six adult males and two adult females) between August 2011 and November 2011 with Trovan Unique® microchips (Trovan Ltd., U.K.). During the period of in-stream monitoring, a further 122 platypuses (59 adult males, six subadult males, four juvenile males, 51 adult females and two juvenile females) were microchipped with Trovan Unique® microchips in the connected waterways of the Inglis River Catchment bringing the total number of animals microchipped since 2007 in the study area to 153 by the end of this study (Figure 3.1).

Observations of platypuses microchipped between August 2011 and August 2013 by the two different antenna types (Trovan®ANT 612 - Units 1, 4, 5 and 6; and Trovan®ANT C600 - Units 2 and 3) were not distinguished for analysis. Water stopped flowing during particular monitoring periods in summer months at each of five sites. Because microchipped platypuses continued to be observed during periods of no flow (Figure 3.2 Site A shows a track across the antenna where the algae is likely to have been worn off by platypus movements), data from these periods was not distinguished for analysis.

The microchip reader units monitored constantly until a microchip is detected, after which monitoring was suspended for a pre-set wait time before continuous monitoring was recommenced. During the two first monitoring periods (which were at Site A), wait times of 0.1, 1 and 5 s were tested on different days. Subsequently, at the other sites, the wait time was set at 10 s. During three monitoring periods (one at Site D, one at Site G,

one at Site J) the antennas from two monitoring units were placed in the same creek within 3 m of each other. Any two microchip recordings of the same platypus at a single site separated by <30 min (from a single unit or from two units in the same creek) were classed as a single platypus observation. The same principle was applied to any number of microchip recordings for the same platypus where consecutive intervals were <30 min.

Data from the monitoring periods reported by (Macgregor *et al.*, 2015) are included in this section. Because a large amount of data was gathered from a single site (Site I), some of the results from this site are presented separately from those of the other sites to allow more detailed analysis of data from this site and to avoid obscuring data from the other sites.

Sunset/sunrise data was downloaded from

http://uk.weather.com/climate/sunRiseSunSet-Wynyard-Airport-ASXX0369

and corrected for daylight saving time using data downloaded from

http://www.dpac.tas.gov.au/divisions/policy/daylightsaving.

Day time was defined as being the period after sunrise and before the next sunset (diurnal). Night time was defined as being the period after sunset and before the next sunrise (nocturnal).

The effect of time of year and platypus identity on platypus behaviour at site I. Time periods for these analyses were based on the investigations into reproductive seasonality in Chapter 4 and reported observations of platypus behaviour in captivity (Hawkins and Battaglia, 2009). For females, 20th September - 24th February (representing the period of expected increased activity during the lead up to the breeding season, the breeding

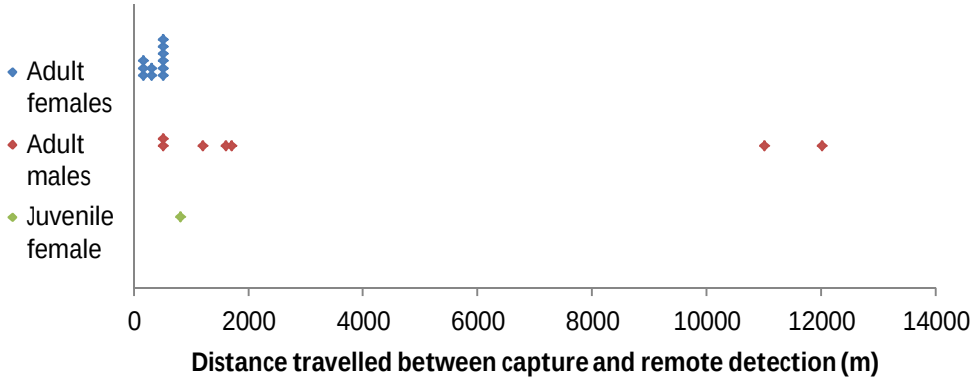
season and early lactation) was compared to 25th February - 19th September. For the male, 20th September - 24th December (representing the period of expected increased activity during the lead up to the breeding season and during the breeding season) was compared to 25th December – 19th September. For Platypuses 71, 72, and 75, type III mixed model ANOVA tests were performed with either the total number of observations, number of daytime observations and number of night time observations per 24 hour period as the dependent factor, time of year (20th September - 24th February versus 25th February – 19th September) as a fixed factor and platypus identity as a random factor. Separate one way ANOVA tests were performed with either the total number of observations, number of daytime observations and number of night time observations per 24 hour period as the dependent factor, and time of year (20th September - 24th February versus 25th February – 19th September) the categorical predictor for each of the females 71, 72, and 75. Statistical analysis of results was performed using Statistica 8.0 (Stat Soft Inc. Tulsa OK, USA). One way ANOVA tests were performed with either the total number of observations, number of daytime observations and number of night time observations per 24 hour period as the dependent factor, and time of year (20th September - 24th December versus 25th December – 19th September) the categorical predictor for the male Platypus 74.

3.3 RESULTS

Over the total of 1,476 monitoring days (sum of the number of monitoring units x number of days in place), 3,622 platypus observations were made of 55 platypuses (25 adult females, one juvenile female, 26 adult males and three subadult males at their respective times of first capture). A total of 2,117 platypus observations were made at Site I during the 548 days of monitoring at that site. The remaining 1,505 platypus

observations were made across the other 17 monitoring sites. Five of the 12 platypuses (42%; one adult female, four adult males) originally captured in 2007-8 and identified with ISO microchips were detected at sites monitored in this study by Units 2 and 3 (three at Site G, two at Site J). Of the 45 platypuses (21 adult females, 21 adult males, three subadult males) captured and microchipped with Trovan®Unique microchips between August 2012 and the respective monitoring periods at sites A – R, 37 (82%; 16 adult females, 18 adult males, 3 subadult males) were detected: 31 only at the site of their capture, five at the site of their capture and at another site and one only at a site where it hadn't been captured. A further 13 platypuses (eight adult females, one juvenile female, four adult males) were detected that had been captured and microchipped in the same time period but at locations other than the remote monitoring. For the 19 platypuses that were observed at sites other than that of their capture, the distances via waterways between the site of capture and the site of detection by in-stream monitoring units are illustrated in Figure 3.5.

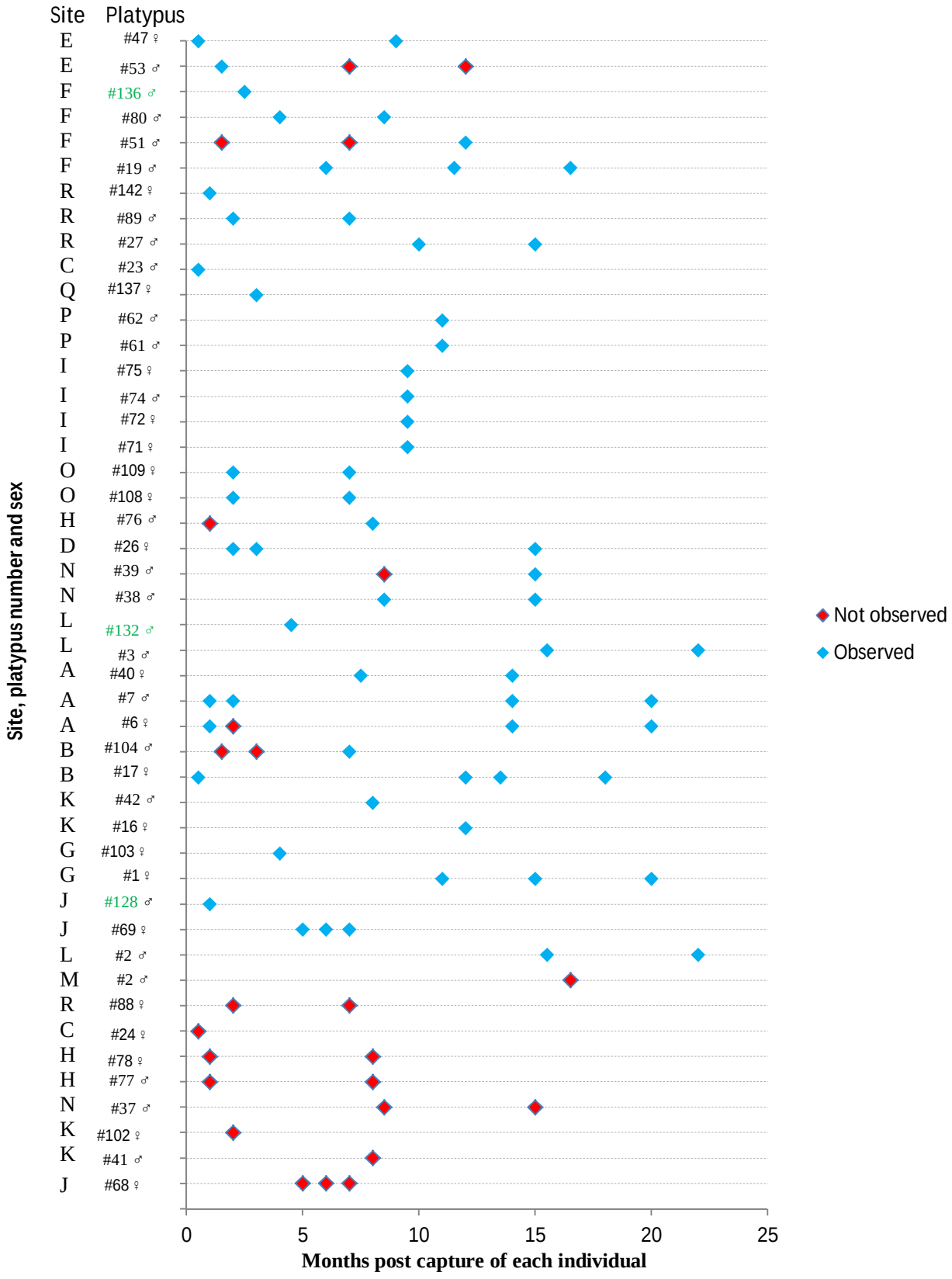
Figure 3.5. The distances via waterways between the site of capture and the site of detection by in-stream monitoring units for platypuses that were detected at sites other than that of their captures.



The two platypuses that travelled 11-12 km between capture and monitoring sites were both adult males. One was only detected once at Site G, the other was detected on three occasions in each of two monitoring periods 20 months apart at Site A. A Mann Whitney U test showed that the distribution of distances travelled by adult females (median=500m) and by adult males (median=1,600m) to be significantly different (U=6, p=0.003)

For each platypus captured at a monitoring site, Figure 3.6 illustrates the time after capture that each monitoring period at that site took place and whether the platypus was observed during that monitoring period. Platypus 2 is depicted twice, once at the site of its capture, where it wasn't detected in the single monitoring period performed, and once at a separate site 1.6km away where it was detected in two monitoring periods. Further details of the observations of Platypuses 71, 72, 74 and 75 are given below. Of the 23 platypuses captured in the Inglis catchment in 2007-2008, four where detected on all of the occasions that antennas capable of detecting ISO microchips were used at their site of capture, and one individual was detected during one of the two such monitoring periods.

Figure 3.6. Platypus presence/absence at monitoring sites during monitoring periods. For each platypus captured at a monitoring site, the time after capture of the mid-point of each monitoring period that took place at that site and whether the platypus was observed is illustrated. Platypus 2 (captured at one monitoring site but only detected at another) is represented twice. Green number = subadult at time of capture.



For the majority of platypuses (59%), detections occurred at a rate up to 0.5/day (Figure 3.7). A Mann Whitney U test showed that the distribution of detection frequencies for adult females (median=0.95 observations/day) and adult males (median=0.27/day) to be significantly different ($U=737$, $p<0.001$). Overall, 720 (20%) of 3,622 platypus observations occurred during the day. At Site I, 604 (29%) of 2,117 platypus observations occurred in the day; at the other sites combined, 116 (8%) of 1,505 platypus observations occurred in the day (Figure 3.8). Further detail on the timings of the observations is illustrated in Figures 3.9 and 3.10, which illustrate the time and date of all platypus observations at Sites A-H/J-R and Site I, respectively.

Figure 3.7. Number of platypus observations per day of monitoring for the age/sex categories of platypuses detected, in each monitoring period that each individual was detected.

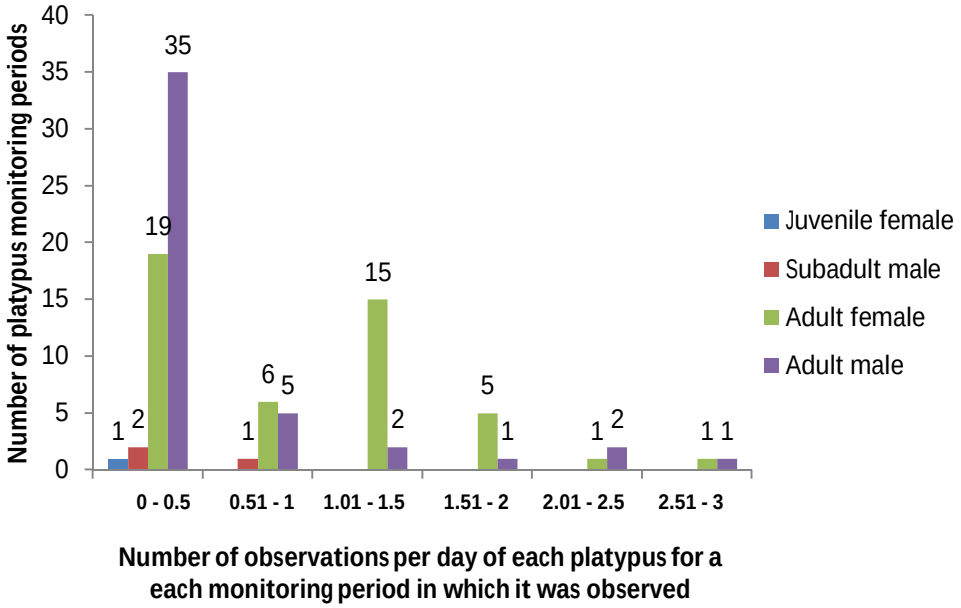
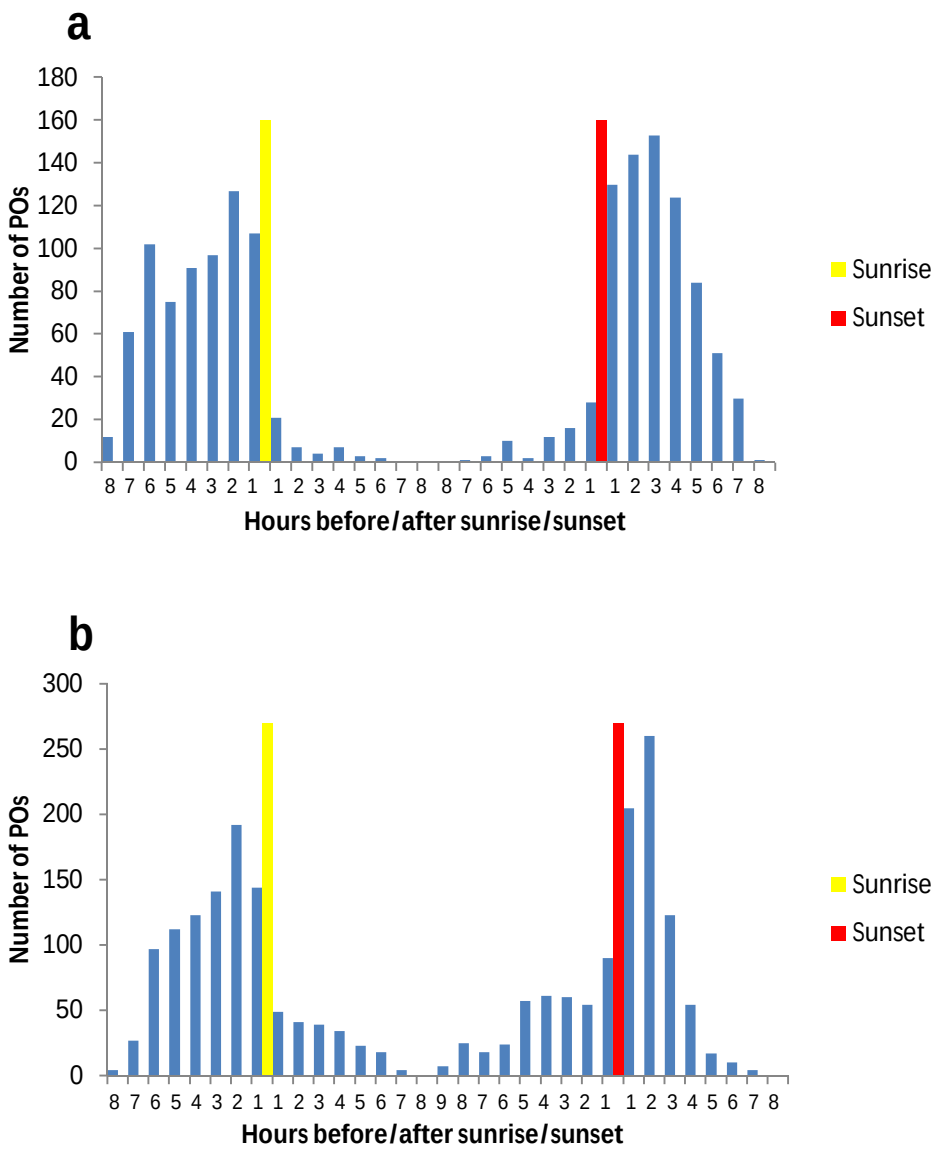


Figure 3.8. Time before/after sunset of platypus observations (POs) at a) Sites A-H and J-R, and b) Site I.



Observations were made of eight platypuses at Site I. The details of these platypuses are given in Table 3.3 and the timings of their observations are illustrated in Figure 3.10. Platypus 72 was not detected between 16/3/2014 and 9/5/2014 (last day of study period). Otherwise, she was regularly detected throughout the monitoring period, with the largest interval between observations of this platypus being 14 days.

Table 3.3. Details of the platypuses detected, over a 548 day monitoring period, by the in-stream microchip reader at Site I.

Platypus	Age	Sex	Date of capture	Location of capture	Number of observations
#71	Adult	Female	2/10/2012	Site I	583
#72	Adult	Female	2/10/2012	Site I	632
#74	Adult	Male	2/10/2012	Site I	214
#75	Adult	Female	2/10/2012	Site I	644
#108	Adult	Female	19/12/2012	Site O†	1
#144	Adult	Male	14/05/2013	Site 88‡	1
#150	Adult	Male	13/08/2013	Site O†	2
#151	Adult	Female	13/08/2013	Site O†	40

† Site O was 550 m from Site I
‡ Site 88 was 650 m from Site I

Figure 3.9. Dates and times of all platypus observations from all monitoring periods at Sites A - H and J - R. Blue lines at top indicate dates when monitoring was occurring at least one of these sites and letters indicate sites where this monitoring was taking place.

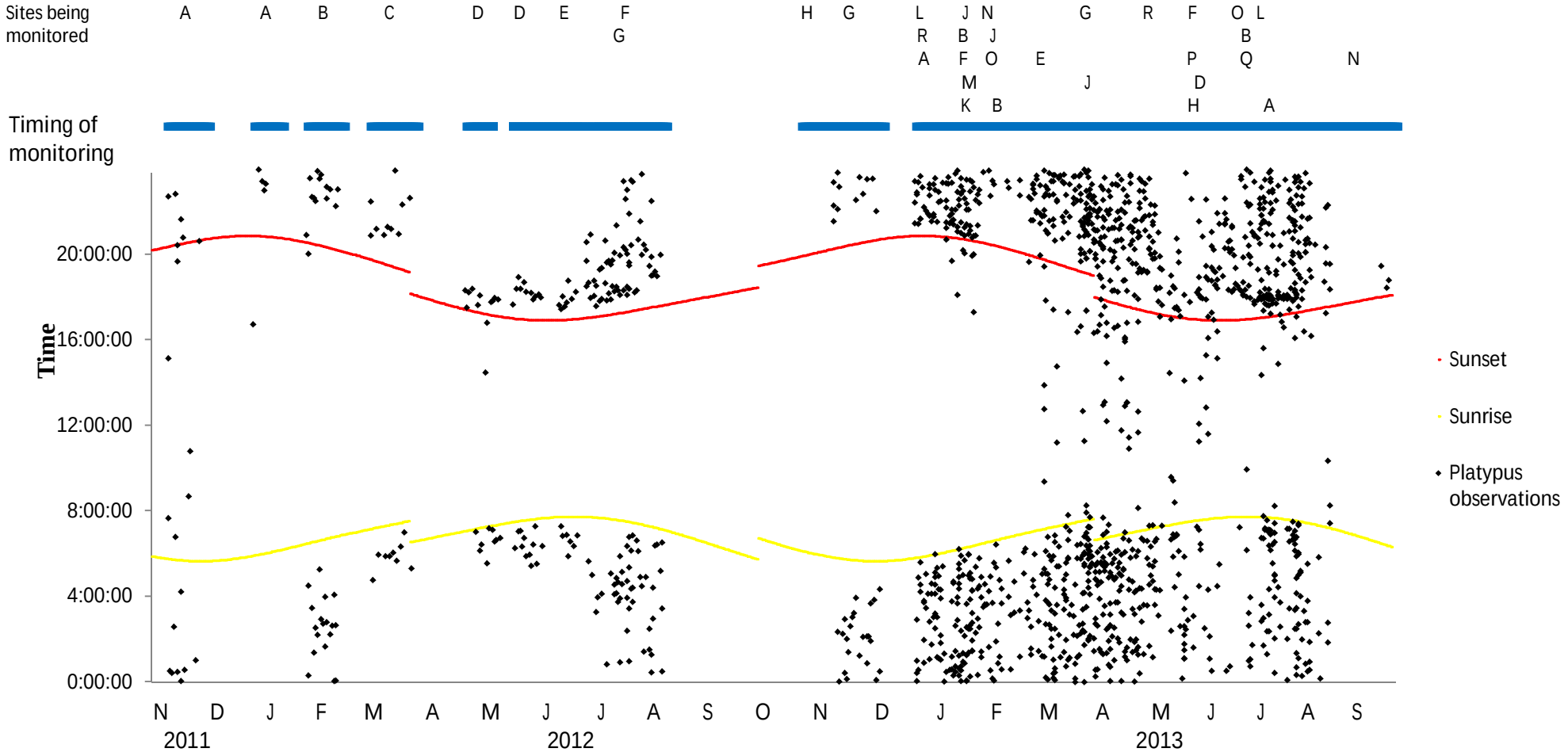
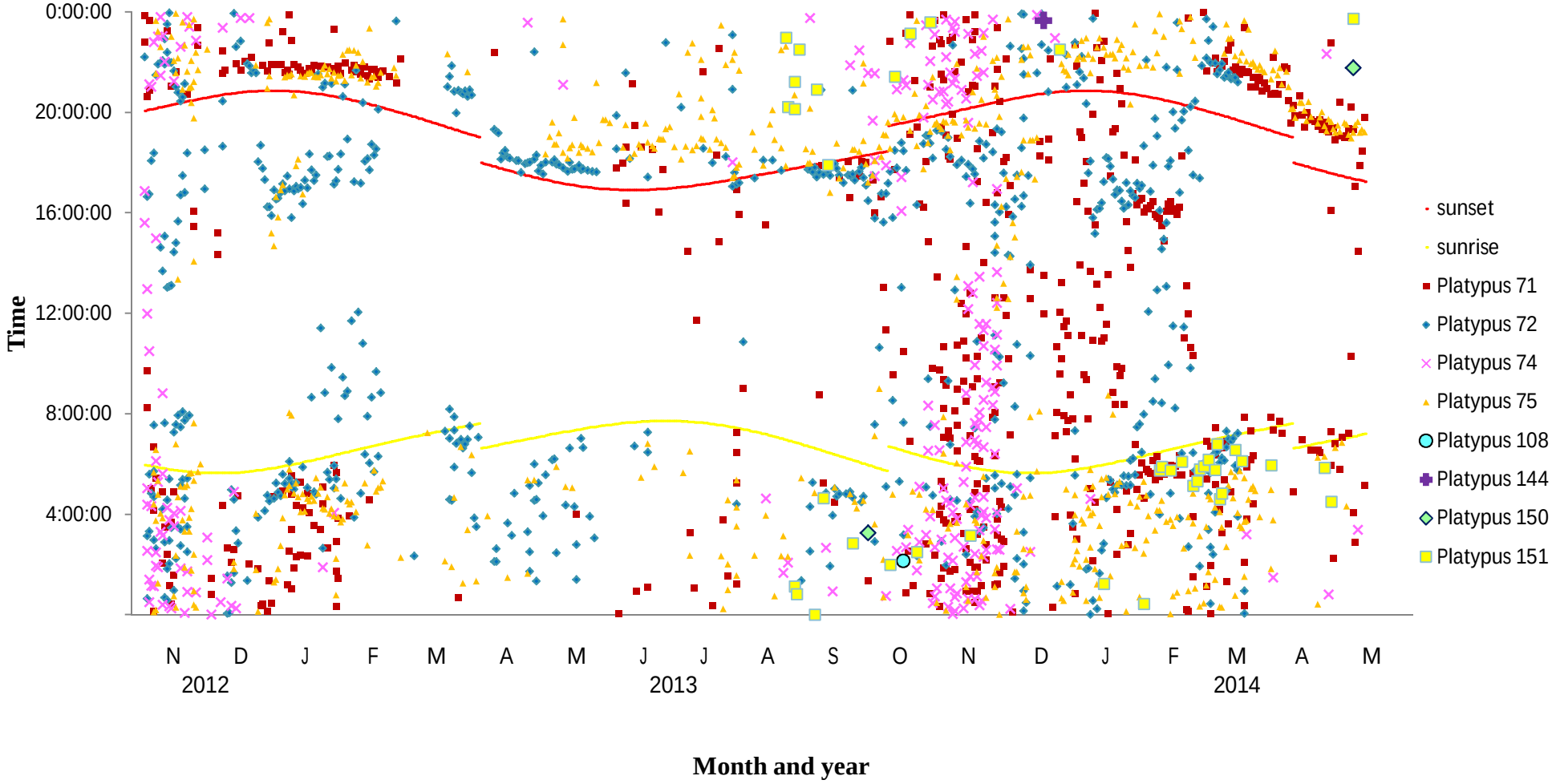


Figure 3.10. Dates and times of all platypus observations from all individuals detected at Site I.



For Platypuses 71, 72, and 75, mixed model ANOVAs with number of platypus observations in a 24 hour period as the dependent factor, time of year (20th September - 24th February versus 25th February - 19th September) as a fixed factor and platypus identity as a random factor showed a significant correlation between time of year and total number of observations (higher between 20th September - 24th February) and a near significant correlation between time of year and number of both nocturnal and diurnal observations of observations (higher between 20th September - 24th February) (Table 3.4). However, there was no effect of platypus identity (Table 3.4). The results of one way ANOVA indicated that the total number of observations as well as the number of diurnal and nocturnal observations per 24 hour period for each of the females 71, 72 and 75 were significantly or highly significantly greater for dates between 20th September to 24th February when compared to the rest of the year (Table 3.5). In addition, the results of one way ANOVA indicated that the total number of observations as well as the number of diurnal and nocturnal observations per 24 hour period for the male 74 were highly significantly elevated for dates between 20th September to 24th December when compared to the rest of the year (Table 3.6).

Table 3.4. Results of mixed model ANOVAs with number of platypus observations (POs) in a 24 hour period as the dependent factor, time of year (20th September to 24th February = 1, 25th February to 19th September = 0) as a fixed factor and platypus number as a random factor for Platypuses 71, 72, and 75 (females) at Site I.

Total number of POs		df Effect	MS Effect	df Error	MS Error	F	p
Fixed	Time of year	1	332.67	2.00	2.81	118.23	**0.008**
Random	Platypus	2	1.86	2.00	2.82	0.66	0.602
Number of diurnal POs							
Fixed	Time of year	1	121.37	2.00	8.93	13.59	0.066
Random	Platypus	2	12.42	2.00	8.94	1.39	0.419
Number of nocturnal POs							
Fixed	Time of year	1	52.16	2.00	3.96	13.16	0.068
Random	Platypus	2	17.05	2.00	3.97	4.30	0.189

Table 3.5. Results of one way ANOVAs between number of platypus observations (POs) and time of year (20th September to 24th February = 1, 25th February to 19th September = 0) for each of Platypuses 71, 72 and 75 (females) at Site I.

Fixed - Time of year	df Effect	MS Effect	df Error	MS Error	F	p
Total number of POs (71)	1	139.64	547	1.50	93.35	**<0.001**
Number of diurnal POs (71)	1	42.56	548	0.43	100.08	**<0.001**
Number of nocturnal POs (71)	1	28.12	547	0.96	29.28	**<0.001**
Total number of POs (72)	1	124.12	547	1.35	91.71	**<0.001**
Number of diurnal POs (72)	1	85.93	547	0.35	246.23	**<0.001**
Number of nocturnal POs (72)	1	3.50	547	0.86	4.06	**0.044**
Total number of POs (75)	1	74.53	547	1.21	61.46	**<0.001**
Number of diurnal POs (75)	1	10.88	547	0.18	60.90	**<0.001**
Number of nocturnal POs (75)	1	28.47	547	1.06	26.73	**<0.001**

Table 3.6. Results of one way ANOVAs between number of platypus observations (POs) and time of year (20th September to 24th December = 1, 25th December to 19th September = 0) for Platypus 74 (male) at Site I

Fixed - Time of year	df Effect	MS Effect	df Error	MS Error	F	p
Total number of POs	1	186.80	547.00	0.86	217.51	**<0.001**
Number of diurnal POs	1	13.32	547.00	0.23	58.34	**<0.001**
Number of nocturnal POs	1	100.35	547.00	0.44	226.57	**<0.001**

Closer examination of the data for each female platypus during and shortly after the two periods when Platypus 74 (adult male) was frequently detected at Site I (starting November in each year), reveals a similar pattern in five of six instances (Figures 3.11 and 3.12). In each of these five instances there were a high number of observations with an irregular diurnal pattern during and shortly after the period of frequent male platypus observations. Following this there was a 12-30 day period with a minimal number of observations. Lastly, there was an extended period with a moderate frequency of observations with a relatively regular pattern. For Platypus 71, an initial period of high number of observations followed by a period of minimal number of observations occurred during November - December 2013 (Figure 3.12). However, instead of proceeding to a period with a moderate frequency of observation with a relatively regular pattern, another period with a high number of observations and an irregular pattern occurred. Platypus 74 was detected only once during this second period of high activity (the presence of a non-microchipped male cannot be excluded), and after five weeks platypus Platypus 71 entered a period of moderate frequency relatively regular observations with no preceding period of minimal observations.

Figure 3.11. Comparing observations of three female platypuses with the presence of Platypus 74 (male) at Site I between early November 2012 and early February 2013. PO = platypus observation.

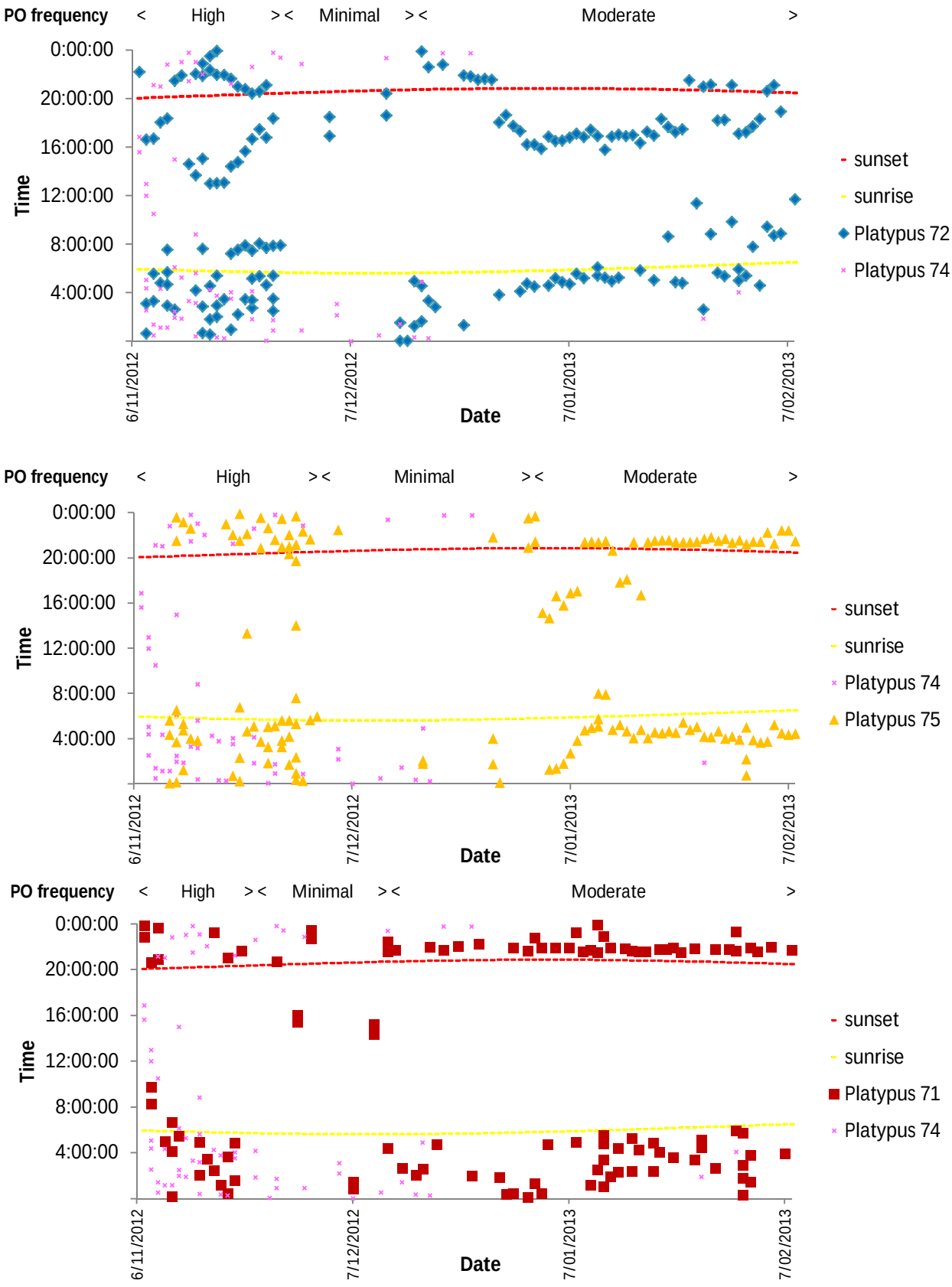
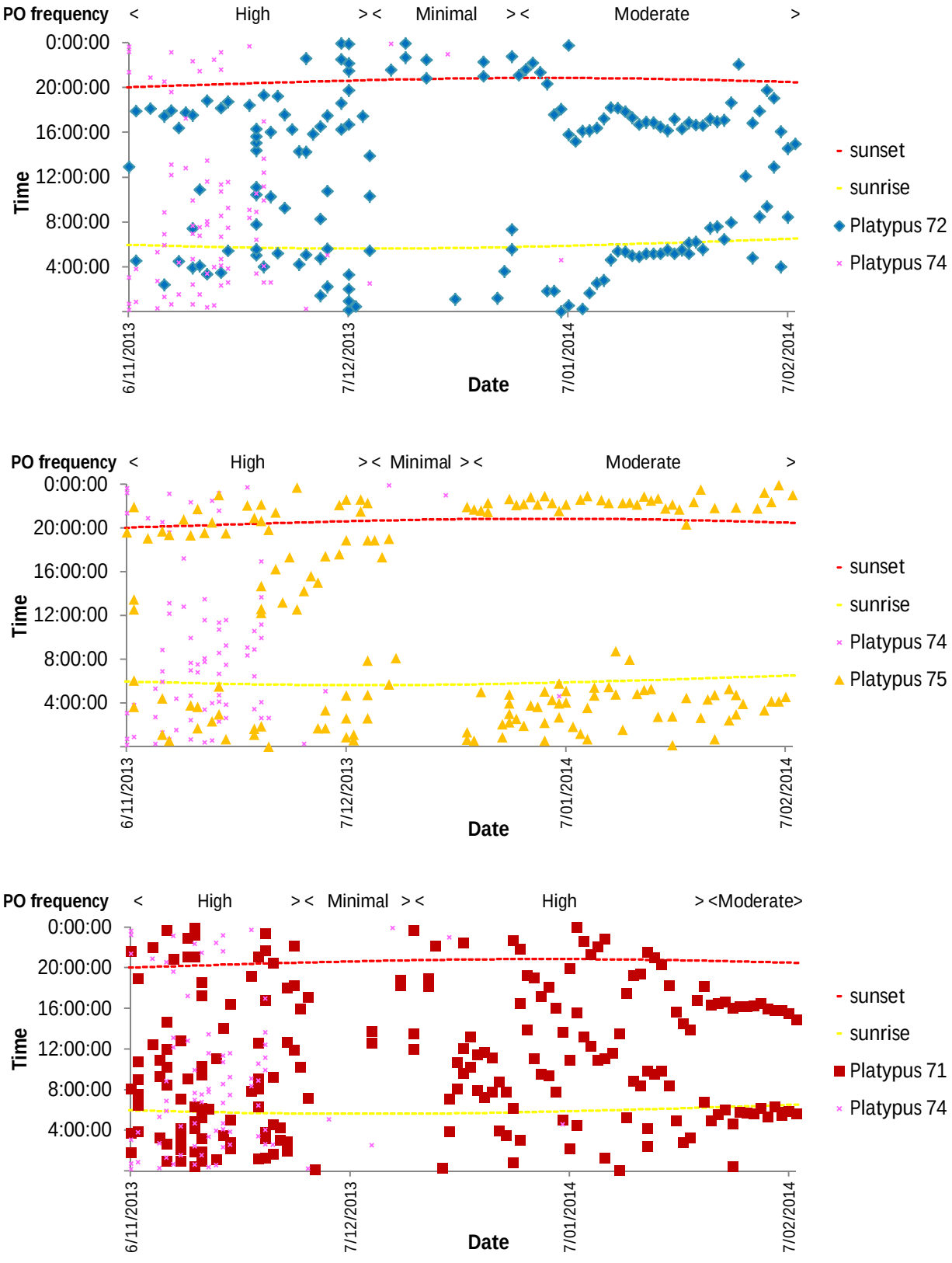


Figure 3.12. Comparing observations of three female platypus with the presence of Platypus 74 (male) at Site I between early November 2013 and early February 2014. PO = platypus observation.



3.4 DISCUSSION

This project demonstrated the continued use of their captures sites by 80% of platypuses captured up to two years previously and 42% of platypuses captured four to six years previously. The number of observations per day was significantly lower for males than for females, suggesting a larger home range for males. For platypuses that were detected at a site other than that of their capture, the distance between the capture and detection sites was significantly greater for males than for females. Only 20% of platypus observations occurred during the day. However, seasonal variations in the number of observations both in the day and in the night, as well individual patterns of observations, were consisitent with breeding behaviour.

Including platypuses microchipped in 2007/2008 and after August 2012, the detection over 30 months of 74% of platypuses captured at the monitoring sites is a higher figure than the recapture rates of both Grant (2004) and Serena and Williams (2013) during live capture/release studies over periods of 8 to 30 years (Table 3.7). Low recapture rates in platypus fieldwork may be related to net avoidance after initial capture (Griffiths *et al.*, 2013) or movement of individuals away from fieldwork sites (Grant, 2004). To some extent, it would appear that instream microchip readers overcome the issue of net avoidance.

Table 3.7. Recapture/remote detection rates for long-term platypus studies.

Reference	Location	Method	Duration of study	Sex	n marked	% recaptures/ remote detections
Grant (2004)	upper Shoalhaven River, NSW	Live capture	~12 years	males females	271 429	36% 51%
Serena and Williams (2013)	Near Melbourne, Victoria	Live capture	~12 years	males females		58% 73%
Serena and Williams (2013)	Wimmera River, Vic	Live capture	Over 8 years	Males Females		38% 31%
Present study	Inglis Catchment, Tasmania	In-stream remote monitoring	30 months	males females	31† 26‡	80% 65%

† Includes 7 males captured during 2007-2008
‡ Includes 5 females captured during 2007-2008

Although it is not possible to calculate home range areas from the monitoring method used in the present study (since the monitoring was carried out at single stations) the data do suggest that the majority of individuals (80%) were resident to the areas surrounding their capture sites. Relating distances to the home range sizes reported by previous studies also suggests that the 13 and four platypuses detected within 0.6 km and 2 km, respectively, of the sites of their captures (Figure 3.5) were also likely to have still been within a stable home range (Grant *et al.*, 1992; Serena, 1994; Gardner and Serena, 1995; Gust and Handasyde, 1995; Serena *et al.*, 1998; Serena and Williams, 2013). However, it seems likely that the eight individuals that were captured at the monitoring sites but not detected by the in-stream antennas should not be considered as being resident to the surrounding areas over the course of the study.

In keeping with the conclusions of Macgregor *et al.* (2015) the results displayed in Figure 3.7 are consistent with variability due to varying position of the antenna within the home ranges of different platypuses and increased use of central parts of the home

range as reported by Gust and Handasyde (1995). The highly significant difference between adult male and female platypuses for the mean number of daily platypus observations for individuals during each monitoring period as indicated by the result of a Mann Whitney U test is consistent with previously reported smaller home ranges in female platypuses when compared to male platypuses (Serena and Williams, 2013) as suggested by Macgregor *et al.* (2015). This is also supported by the highly significant difference between males and females in the distance between capture and detection sites for those platypuses that were detected at a site other than that of their capture.

The results, illustrated in Figures 3.8 to 3.10 relating to time of day and time before/after sunrise/sunset of platypus observations are consistent with the findings of previous studies that platypuses are more active at night (Gust and Handasyde, 1995; Otley *et al.*, 2000; Bethge, 2002). However, Figure 3.10 illustrates that the time before/after sunrise/sunset of platypus observations at Site I appeared to vary with time of year, in particular relating to the breeding season, and this should be an important consideration of any investigation into this aspect of platypus ecology.

The 548 day monitoring period at Site I allowed analysis of daily and seasonal temporal activity patterns. While at Sites A-H and J-R there were only short monitoring periods and relatively few monitoring days in spring (mid-August and December), at Site I, there was continuous monitoring, through all seasons, mostly of the same four platypuses (three female, one male) that had been captured there (Table 3.3; Figure 3.10). Records of the male Platypus 74 at this site occurred almost exclusively during spring (September-November. The number of observations (both diurnal and nocturnal) of the female Platypuses 71, 72, and 75 increased during the time that Platypus 74 was

detected. The results of the ANOVA tests reinforce this apparent increase in activity at this time. In combination with the physiological evidence of reproductive seasonality (Chapter 5), these changes in activity patterns suggest marked changes in behaviour around the breeding season in November/December. The high activity/quiescence pattern observed (Figures 3.11 and 3.12), is similar to patterns of behaviour reported around the breeding season for captive platypuses in New South Wales (Hawkins and Battaglia, 2009). In their study over six years of three females and one male, the authors observed that the breeding season usually started in September/October with increased mate-seeking activity in males. In successful breeding years, females initially avoided males until they became receptive for 4-6 days, when they would change their behaviour to coincide with that of the male. After mating, females remained relatively active visiting (a) nesting burrow(s) but with some extended burrow visits, and then became intensely active collecting nesting material and taking it to the nesting burrow. Within 15-21 days of mating, females retired to their burrows for 20±2 days, spending only occasional short periods out of the burrows. It was assumed that the eggs were laid shortly after retirement, followed by incubation. From day 20±2 to 45±5 after initial retirement to the nesting burrow, females spent gradually more time out of the burrow. From day 45±5 onwards, the females would visit the nesting burrow only every second day, resting elsewhere between visits. In unsuccessful breeding years, females displayed breeding events including mating, nest selection, collection of nesting material and initial retirement, but no live offspring emerged from the burrow. In one breeding season, a female was observed to have two receptive periods a month apart (Hawkins and Battaglia, 2009).

The activity patterns observed for the three free-ranging female platypuses monitored at Site I suggest that the period of increased observation frequency with an irregular pattern (early to mid-November) is consistent with pre-mating activity, post-mating burrow selection and nesting material collection. The following 2-4 week quiescent period is consistent with initial retirement, followed by a period of moderate numbers of observations at more regular intervals likely to be consistent with the early lactation phase. Observations of Platypus 71 in 2013 followed this pattern similar until the end of the initial retirement phase. At this stage, instead of a phase of moderate, relatively regular observation frequency, a period of frequent observations at irregular intervals, similar in nature to that in November, returned.

It seems likely that the frequency of platypus observations reflected the level of activity in these platypuses, and that between platypuses 71, 72 and 75, five pregnancies occurred that were successful at least to around 20 days after initial retirement to the burrow, but that platypus 71 did not successfully breed in 2013. Although platypus 75 has since been detected at this site, the absence of any observations of this female between 16/3/2014 (~ three months after the apparent date of her retirement to the nesting burrow) and 9/5/2014 (last day of study period) may indicate a failure to raise young until emergence in this year. In 2013, observations of the male platypus and the periods of minimal observations in the females occurred later than in 2012. This is consistent with observations in other seasonally breeding species that the timing of breeding can vary with environmental factors (Perrins, 1965; Davies and Lundberg, 1985; Green, 1988).

Findings of this study suggest that at suitable sites, in-stream antennas can play an important role in assessing continued use of an area, survivorship and reproductive success to the point of emergence, with advantages over other research methods in terms of increased detection rates, reduced labour, and reduced number of live captures required. Additionally, the extensive temporal activity data that can be collected has shown to be valuable as a method of recording activity patterns around breeding. To date, studying reproduction in wild platypuses has relied on chance live captures of juvenile platypuses, assessing milk let down on injection of oxytocin, reproductive hormone analysis and ultrasonography (Grant *et al.*, 1983; Connolly and Obendorf, 1998; Jakubowski *et al.*, 1998; New *et al.*, 1998; McLachlan-Troup, 2007; Serena *et al.*, 2014). By contrast, in-stream microchip readers do not rely on capture of individuals at specific times of year, overcomes the problems associated low recapture rates and minimises disturbance due to live capture. From a single capture, in-stream microchip readers can gather data from individuals for as long as they continue to use same suitable location, which, from the results of this the study, appears likely to be measured in years for many platypuses.

Chapter 4.

Immunogenetics

4.1 INTRODUCTION

Genetic diversity at loci concerned with fitness is an important part of the ability of a wild population to adapt to changes in its environment including climatic events, disease and pollution (Frankham, 1995; Reed and Frankham, 2003; Holderegger and Wagner, 2006; Markert *et al.*, 2010). The effects of environmental and genetic factors in population declines may be difficult to separate and "understanding of the relationship between diversity and long-term population viability is limited" (Markert *et al.*, 2010) p11). Frankham (1995) suggested that some species extinctions attributed to environmental or anthropogenic factors may actually have been due to interactions between genetic and non-genetic factors.

Lande (1988) suggested that environmental and demographic factors generally lead to extinction before genetic factors have time to have an effect. However, evidence from more recent studies in both laboratory and field situations have supported the idea that lack of genetic diversity can affect species' survival (Frankham, 2003). For example, Eldridge *et al.* (1999) found low levels of genetic variation in small island populations of black footed rock wallabies (*Petrogale lateralis*) which was associated with reduced fitness. Additionally Saccheri *et al.* (1998), reported that low levels of heterozygosity in relatively isolated populations of wild Glanville fritillary butterflies (*Melitaea cinxia*) were associated with an increased rate of local population extinctions. In laboratory tests, Markert *et al.* (2010) observed reduced fitness in the low genetic diversity populations of the crustacean *Americamysis bahia* in both normal and stressful environments. Reed and Frankham (2003) reviewed 34 studies that measured a component of fitness as well as heterozygosity, and/or heritability and/or population size. They concluded that genetic variation is positively and significantly correlated

with population fitness. Genetic diversity is generally positively related to population size (Frankham, 1996), and so the effects of low genetic diversity are likely to be seen increasingly as the size of a population of a threatened species declines. Consistent with this, population size appears to be correlated to fitness (Reed and Frankham, 2003). Natural fluctuations in population size that occur in wildlife species therefore mean that genetic diversity may be a better measurement of fitness than population size at any particular point in time (Reed and Frankham, 2003).

Laboratory and *in situ* evidence indicates that low genetic diversity can affect the ability of wild populations to adapt to a new infectious challenge. In a laboratory situation, Spielman *et al.* (2004) observed that loss of genetic diversity in populations of *Drosophila melanogaster* resulted in reduced resistance to infectious disease due to loss of specific resistance alleles. Also experimentally, Pearman and Garner (2005) found an increased mortality risk from an emerging viral pathogen in populations of the Italian agile frog (*Rana latastei*) with reduced genetic diversity. Furthermore, Whiteman *et al.* (2006) reported higher parasite abundances in populations of Galapagos hawks (*Buteo galapagoensis*) with lower genetic diversity, which significantly correlated with lower antibody levels. A high prevalence and diversity of potentially pathogenic infections has also been reported in the genetically-depauperate Florida panther (*Felis concolor coryi*) (Roelke *et al.*, 1993).

Research into the effects of genetic diversity on the impacts of disease on wildlife populations has focussed on genes of the major histocombatibility complex (MHC). The MHC is a multigene family that is central to the vertebrate immune system (Piertney and Oliver, 2005). In the human genome the MHC is the most gene dense region and is

found on chromosome 6 (The MHC Consortium, 1999). It was discovered because of its role in alloreactivity, but has a broader immune system role with over 40% of the genes in the human MHC having immune function (The MHC Consortium, 1999; Vandiedonck and Knight, 2009).

Genes of the MHC are divided into four subgroups (Classes I-IV), with antigen presenting genes found in subgroups I and II (The MHC Consortium, 1999; Acevedo-Whitehouse and Cunningham, 2006). The products of the MHC class II genes combine to form a cell surface heterodimer, consisting of an α and β chain (encoded by A and B genes, respectively). This heterodimer molecule binds processed pathogenic peptides and presents them on the cell surface to T helper cells (Klein, 1986). These Class II genes are expressed in all antigen presenting cells, such as macrophages and dendritic cells, and are generally highly polymorphic. The extreme polymorphism of the classical MHC genes is thought to be a result of the continual conflict between the immune system and infectious pathogens, and to be associated with evolutionary fitness (The MHC Consortium, 1999; Piertney and Oliver, 2005; Vandiedonck and Knight, 2009). It is for this reason that classical MHC genes are considered suitable subjects for immunogenetic research in relation to population conservation (Acevedo-Whitehouse and Cunningham, 2006).

There has been extensive genetic study of the platypus, including sequencing of the whole genome (Warren *et al.*, 2008). Studies examining gene flow between and within populations have sequenced mitochondrial DNA loci and groups of polymorphic microsatellite loci (Kolomyjec *et al.*, 2009; Gongora *et al.*, 2012; Furlan *et al.*, 2013). Immunogenetic diversity has been investigated by a single study (Lillie *et al.*, 2012).

Unlike many species, including humans, the platypus genome contains only two classical MHC class II genes (Dohm *et al.*, 2007) consisting of the α chain gene DZA and the β chain gene DZB (Belov *et al.*, 2003; Dohm *et al.*, 2007). Primers designed to amplify the MHC class II DZB gene have previously been used to characterise diversity in a number of mainland and Tasmanian platypuses (Lillie *et al.*, 2012). High and comparable levels of diversity at this locus were observed in platypuses from the mainland and from Tasmania (Lillie *et al.*, 2012), but platypuses from Kangaroo Island displayed a lower DZB diversity and King Island platypuses were completely monomorphic at this locus (Lillie *et al.*, 2012).

The present study investigated the level of genetic diversity at the MHC class II DZB gene, as well as the distribution of alleles of the same gene, for platypuses in the Seabrook Catchment. These data will add to the baseline data reported by Lillie *et al.* (2012) and contribute to the overall population health assessment of platypuses in this catchment (Chapter 8).

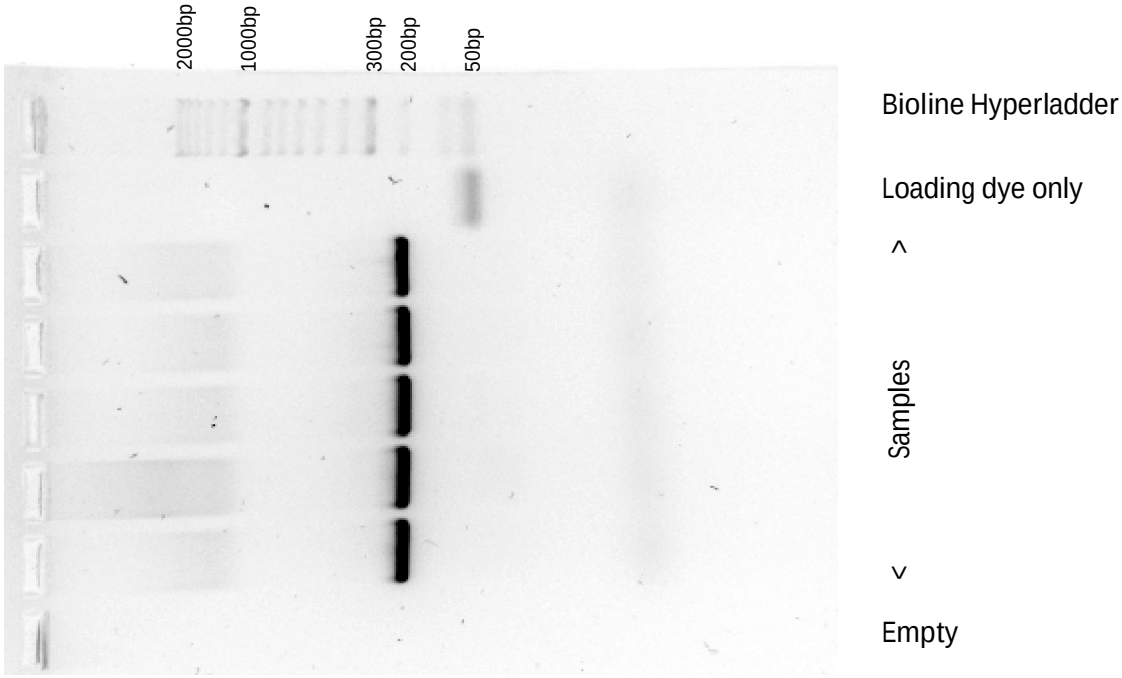
4.2 METHODS

A small (~2 x 2 mm) piece of skin from the interdigital webbing of the right hind foot was collected aseptically under anaesthesia from 24 platypuses captured in the Seabrook Creek Catchment (Section 2.2).

DNA extraction was performed using reagents from the Extract-N-Amp™ Tissue PCR Kit (Sigma Aldrich, St Louis, MO, USA). Tissue Preparation Solution and Extraction solution were mixed in a 1:4 ratio and a piece of the skin clip from each platypus (~1 x 1 mm in size) was incubated in 50µl of the resulting solution at room temperature for 10

min. Following this, the sample/solution was heated to 95°C for 3 min before 40µl of Neutralisation Solution was added. PCR amplifications were performed in 20µl consisting of 10µl MyTaq™ HS mix (Bioline Australia Pty Ltd, Sydney, NSW), 1µl F primer (10µM), 1µl R primer (10µM), 6µl water and 2µl extraction product. PCR primers used were those described by Lillie *et al.* (2012). PCR amplification steps were as follows: 45 cycles of 95°C for 15 s, 60°C for 15 s and 72°C for 10 s. 2µl of loading dye was added to each PCR product and 10µl of each resulting solution was added to a well in a 2% agarose gel made with SYBRSafe™ DNA Gel Stain (Invitrogen, Carlsbad, CA, USA) and run in Tris-acetate-EDTA (TAE) buffer. PCR No Template Controls (NTCs) were run to check for any contaminating products, and fragments were sized against 2µl of DNA HyperLadder™ II (Bioline Australia Pty Ltd, Sydney, NSW). Gel electrophoresis was undertaken at 90V for 40 min. PCR fragments were compared visually with Hyperladder II UV irradiation, demonstrating the presence of DNA fragments of the size expected for the MHC class II DZB gene with primers (199 base pairs; Figure 4.1).

Figure 4.1. Colour inverted image of PCR products under ultraviolet light after gel electrophoresis (contents of each well indicated to right, size of selected Bioline Hyperline II markers indicated at above). **Photo: James Marthick.**



PCR products were excised from the agarose gels and the PCR products purified using Qiagen Gel Extraction Kits in the Qiacube robotic workstation (Qiagen, Venlo, Limburg, Netherlands). The concentration of DNA in each purified PCR product was measured using a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). The amplicons were then ligated into the pGEM®-T Easy Vector (Promega, Madison, WI, USA). Ligations were performed in 10µl volumes, consisting of 1µl vector and 1µl DNA ligase added to water, buffer and purified PCR product (at volumes dependent upon the concentration of each purified PCR product). After overnight incubation at 4°C, 3µl of each ligation product was added to 50µl of JM 109 High-Efficiency Competent *Escherichia coli* cells (Promega, Madison, WI, USA). The cells were then heat shocked for 45-50 s at 42°C, to allow entry of the vector through

the cell walls and into the cells, and then rested for 2 min on ice. 500µl Super Optimal broth with Catabolite repression (SOC) medium was then added to the cells before incubation for 1 h at 37°C. 100µl of each culture was then plated out on Agar plates with 100µl Isopropyl β-D-1-thiogalactopyranoside (IPTG 0.1M) and 20µl 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (XGAL 20 mg/mL in Dimethyl formamide). After overnight incubation at 37°C, 10 white colonies, the colour indicating the presence of the vector with inserted DNA, were picked from each plate and were separately re-cultured overnight at 37°C in 3ml Lysogeny broth (L-broth) on a shaker. *E. coli* cells were pelleted by centrifugation and plasmid DNA was extracted from the cells using Qiaprep Spin Miniprep Kits (Qiagen, Venlo, Limburg, Netherlands) in the Qiacube robotic workstation (Qiagen, Venlo, Limburg, Netherlands). Purified plasmid DNA was quantified using a NanoDrop™1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). 1000 to 1400ng of purified plasmid DNA from each clone was sent for Sanger sequencing at the Australian Genome Research Facility, Melbourne Node.

For the purposes of assessing the distribution of alleles, the Seabrook Creek Catchment was divided into upper, middle and lower parts based on distances from the northern and southern most points of the catchment. Sequences were aligned and compared using BioEdit (Hall, 2004). Allele frequency, gene diversity and inbreeding coefficient were calculated using FSTAT (Goudet, 1995). Consistent with the method of Frankham (1998) and Seymour *et al.* (2001), the effective inbreeding coefficient (F_e) was calculated as $F_e = 1 –$ (observed DZB heterozygosity / DZB heterozygosity in reference population), where the reference population was the platypuses in New South Wales sequenced by Lillie *et al.* (2012). The phylogenetic tree was created using MEGA

(Kumar *et al.*, 1994). The following MHC Class II genes retrieved from Genbank were also included in the phylogenetic analysis:

- short-beaked echidna (*Tachyglossus aculeatus*) (TaacDZB1 [AY288075], TaacDZB2 [AY288076], TaacDZB3 [AY288077], TaacDZB4 [AY288078]) (Belov *et al.*, 2003),
- human (HosaDRB3 [NM_022555]),
- red-necked wallaby (*Macropus rufogriseus*) (MaruDAB1 [M81624] and MaruDBB [M81625].

4.3 RESULTS

Reliable results were obtained from 18 (seven adult males, eight adult females, two juvenile males, one juvenile female) of the 24 platypuses tested. Of the remaining six individuals, two had evidence of contamination (with three previously reported MHC class II DZB gene alleles sequenced). Results from the other four platypuses that were excluded contained multiple sequences of which no more than two were previously reported alleles, but a cause for this was not determined.

The alleles identified in each platypus are shown in Table 4.1. Twelve alleles were identified. Of these, 10 had been identified by Lillie et al. (2012), including eight from Tasmania. The two previously unreported sequences were given the names OranDZB*58 and OranDZB*59 and Genbank accession numbers KP857996 and KP857997. The frequency of observation of each allele is illustrated in Figure 4.2. The observed DZB heterozygosity was 56% and gene diversity 0.895. The inbreeding coefficient and effective inbreeding coefficient at this locus were 0.38 and 0.43,

respectively. Figure 4.3 illustrates the position of the sequenced MHC class II DZB alleles within the known phylogenetic tree for this gene (Lillie *et al.*, 2012).

Table 4.1. Platypus (*Ornithorhynchus anatinus*) MHC Class II DZB allele names of sequences identified in each platypus from the Seabrook Creek Catchment, Tasmania. $^{+}$ Alleles not previously reported from Tasmanian platypuses.

Platypus number	Allele 1	Allele 2 (unless homozygous for Allele 1)
10	OranDZB*2	
12	OranDZB*34	
13	OranDZB*32^{+}	OranDZB*47^{+}
58	OranDZB*17	OranDZB*57
59	OranDZB*17	OranDZB*58
94	OranDZB*34	
95	OranDZB*2	OranDZB*58
97	OranDZB*17	OranDZB*57
124	OranDZB*9	
125	OranDZB*34	OranDZB*52
127	OranDZB*9	
146	OranDZB*13	OranDZB*17
152	OranDZB*2	
153	OranDZB*2	OranDZB*59
154	OranDZB*2	
155	OranDZB*59	
156	OranDZB*2	OranDZB*17
158	OranDZB*28	OranDZB*34

Figure 4.2. Allele frequency at MHC Class II DZB locus in the Seabrook Creek Catchment.

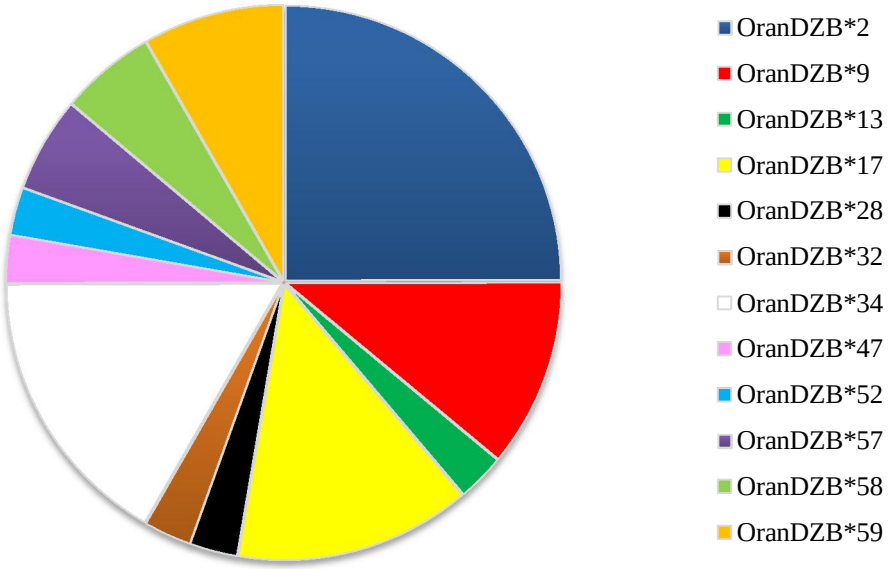
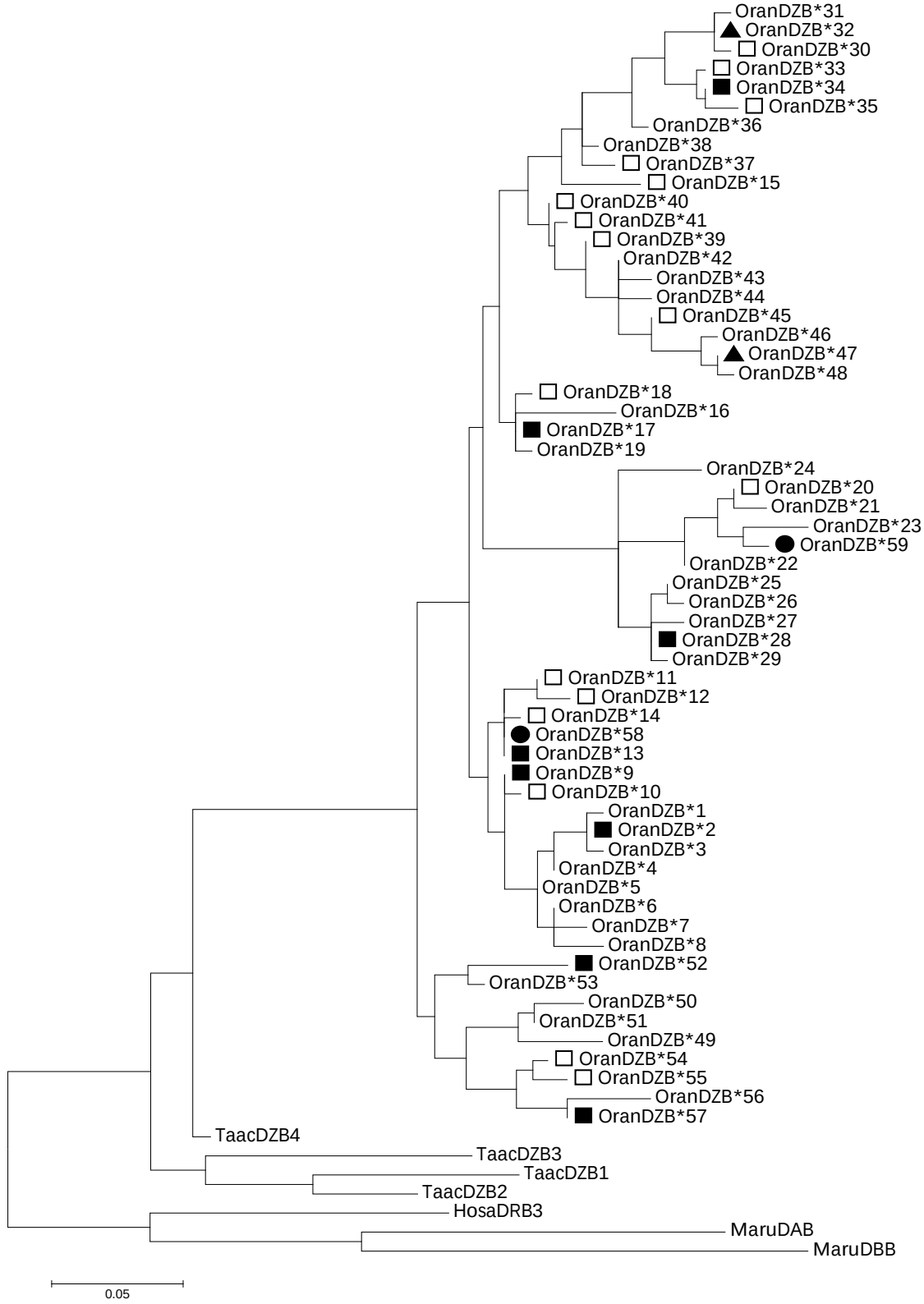


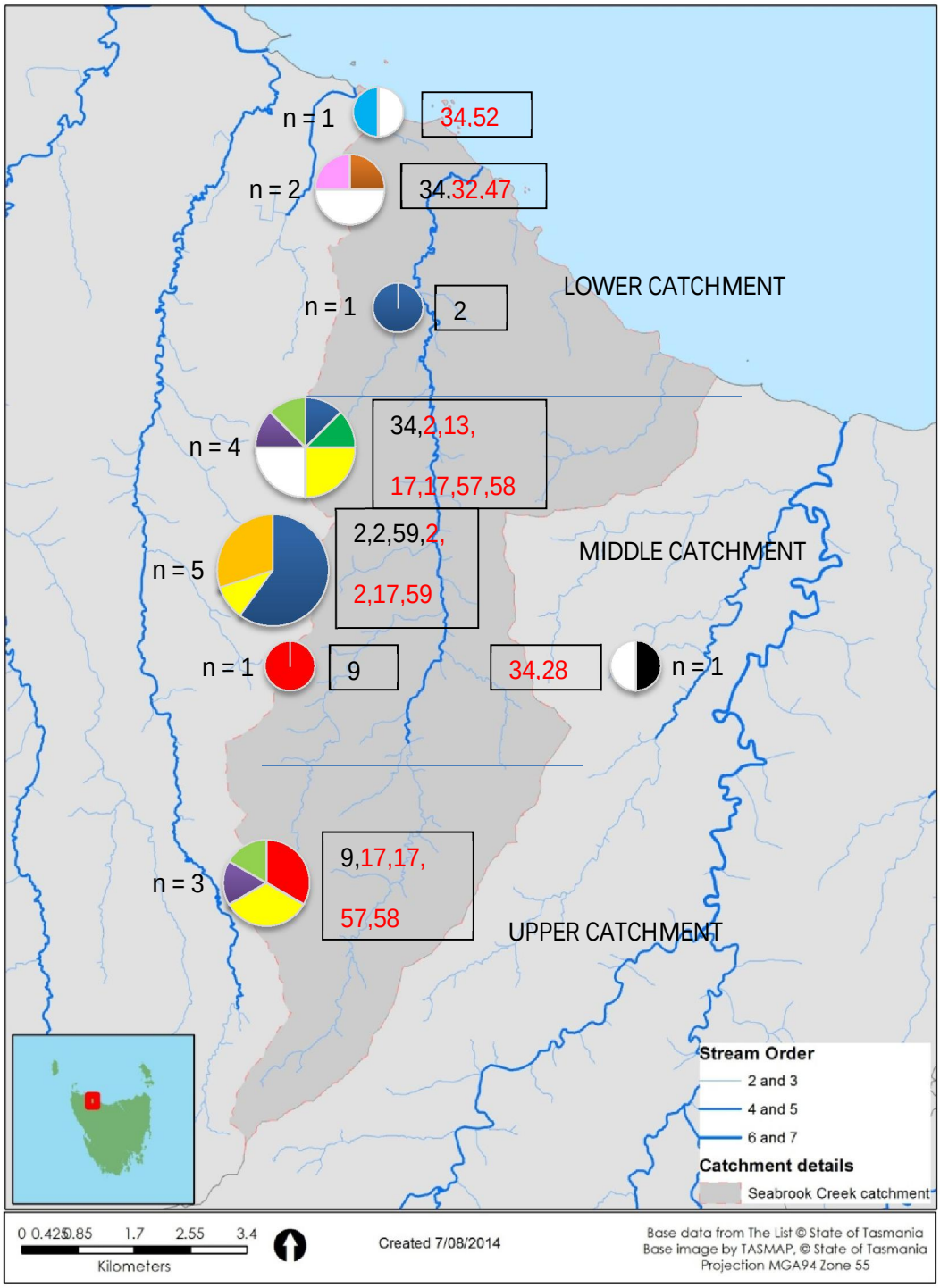
Figure 4.3. Phylogenetic tree for MHC Class II β chain gene. Filled shapes – alleles observed in the Seabrook Catchment that have: i) been previously observed in Tasmania (squares), ii) been previously observed only in mainland states (triangles), or iii) not been previously observed (circles). Unfilled squares - alleles not observed in Seabrook Catchment that have been previously observed in Tasmania.



In addition to the alleles listed in Table 4.1 a sequence of 162 base pairs was cloned twice in Platypus 58 and once in Platypus 12. This sequence was homologous with DZB alleles at ~75% of base pairs, except that a base pair deletion was apparent at position 85, resulting in a frame shift of the remainder of the sequence.

There was reasonable geographic distribution of alleles within the catchment (Figure 4.4). All of the alleles present in the upper part were present in the middle part and two of the five alleles present in the lower part were also present in the middle part of the catchment. The numbers of homozygous platypuses in the lower, middle and upper parts of the catchment were two out of four (50%), five out of 11 (45%), and one out of three (33%), respectively.

Figure 4.4. Capture locations of platypuses in the Seabrook Creek Catchment from which alleles were sequenced. Allele names have been transposed by removing the prefix "OranDZB*". Red numbers are alleles from heterozygous platypuses. Black numbers are from homozygous numbers. Rectangles indicate fieldwork locations. Pie charts illustrate gene frequencies at adjacent sites according to key in Figure 4.2; areas correspond to capture numbers at each location (adjacent n value).



4.4 DISCUSSION

This study detected ten previously identified alleles and two previously unreported alleles at the MHC Class II locus in platypuses from the Seabrook Catchment. Alleles were reasonably well distributed geographically through the catchment. The observed genetic diversity at the MHC Class II locus was lower than those in larger river catchments studied by Lillie et al. (2007) but higher than those in two island populations.

There are many examples of low MHC diversity in threatened wildlife populations (Smulders *et al.*, 2003; Siddle *et al.*, 2007; Kennedy *et al.*, 2011), although the connection between MHC diversity and a population's ability to respond to infection is not completely clear. In some cases, a relationship between low genetic diversity and high disease prevalence has been suggested and later questioned. This was the case for the cheetah, whose homozygosity and MHC loci was presented as a cause for high rates of infectious disease in the species (O'Brien *et al.*, 1985). However, rates of infection were subsequently found to be lower in wild cheetahs than in captive cheetahs, despite having the same lack of genetic diversity (Munson *et al.*, 2005). Environmental factors including stress were considered to be important contributors to the high rate of infectious disease in captive animals (Terio *et al.*, 2004; Munson *et al.*, 2005). In the case of the Tasmanian devil (*Sarcophilus harrisii*), Siddle *et al.* (2007) concluded that a low level of MHC Class I diversity allowed the emergence of devil facial tumour disease (DFTD). However, Lane *et al.* (2012) found no significant differences in MHC class I copy number between 22 diseased devils and 29 healthy controls. Acevedo-Whitehouse and Cunningham (2006) recognised the reasons for this approach in wildlife immunogenetic studies, but noted that MHC is not involved in all immune

responses. They suggested that in some situations, results may be improved by assessment of a broader range of genes with immune function, or by targeting the gene(s) assessed to ones related to specific threatening infections (Acevedo-Whitehouse and Cunningham, 2006).

Threatened populations of some species have recovered in size despite low MHC diversity. For instance, the Scandinavian population of Eurasian beavers (*Castor fibre*) increased in size from ~100 to ~15,000 individuals during the 20th century despite low if any MHC polymorphism (Babik *et al.*, 2005). It has been suggested that most infections do not directly cause death but lead to small changes in an individual's health. As a result, in minimally-impacted populations, protection against these infections by MHC genes may be most important for intraspecific competition, leading to relative stability in population size. However in depleted populations there may be less intra-specific competition, so a small overall reduction in health of individuals in the population as a result of low MHC diversity may not affect population growth if other threatening factors are reduced or removed (Babik *et al.*, 2005). Low MHC diversity in such recovering populations may, however, make them susceptible to introduction of more serious disease, or less able to adapt to environmental change due to mildly reduced individual health (Babik *et al.*, 2005). This is one reason for the importance of this project in gathering of data relating to immunogenetic diversity as well as the prevalences of a broad range of infections in the same wild population (Chapter 7).

In a similar pattern to the previously reported DZB alleles from Tasmanian platypuses, the alleles sequenced from platypuses in the Seabrook Creek Catchment were well distributed across the known phylogenetic tree for this gene (Lillie *et al.*, 2012). Details

of genetic diversity at this locus in different studies are shown in Table 4.2. The number of alleles observed in Tasmania in a previous study may have been higher than that in this study in part because Lillie *et al*. (2012) examined platypuses were sampled from three separate systems of connected waterways, with sites in both the north and south of the state. However, 21 of the platypuses sampled from New South Wales were from the Shoalhaven Catchment (Lillie *et al*., 2012) which suggests a real difference in allele numbers between this catchment and the Seabrook Creek Catchment. The inbreeding coefficient (0.38) and effective inbreeding coefficient (0.43) were of similar magnitude, and were higher than the inbreeding coefficients of all of the populations of studied by Lillie *et al.* (2012) with the exception of the monomorphic population on King Island.

There is evidence that smaller populations of wild species have lower genetic diversity (Frankham, 1996; Montgomery *et al.*, 2000). There is also evidence that river catchments contain relatively isolated populations of platypuses, with only a slow influx/outflow of genes (Gongora *et al.*, 2012; Furlan *et al.*, 2013). In the context of the overall platypus population health assessment, the number of alleles found in the Seabrook Creek Catchment may imply that the likelihood of this population (as defined in Section 1.5) being able to respond to new infectious challenges is intermediate to that in the Shoalhaven Catchment and King Island populations. It seems likely that the lower number of alleles in the Seabrook Creek Catchment and the higher values for inbreeding coefficient and effective inbreeding coefficient compared to those studied by Lillie *et al.* (2012) in NSW and Tasmania were at least in part a consequence of genetic diversity loss due to small population size.

Table 4.2. Summary of genetic diversity in platypus MHC class II DZB across different study locations. † 21 of the platypuses sampled from New South Wales were from the Shoalhaven catchment. ‡ three separate systems of waterways in Tasmania.

Location	Number of individuals	Number of alleles	Observed hetero-zygostiy	Expected hetero-zygosity	Gene diversity	Inbreeding coefficient	Reference
Kangaroo Island	5	5	100%	76%		-0.38	Lillie *et al.* (2012)
King Island	9	1	—	—		—	Lillie *et al.* (2012)
New South Wales †	24	32	96%	98%		0.02	Lillie *et al.* (2012)
Tasmania ‡	23	26	78%	97%		0.18	Lillie *et al.* (2012)
Seabrook Creek Catchment, Tasmania	16	12	56%	86%	0.895	0.38	Present study

The distribution of alleles and homozygotes between upper, middle and lower parts of the catchment suggests that there is little isolation within the population. The only possible exception was one location (consisting of two fieldwork sites ~250 m apart, either side of a farm dam) in the middle part of the catchment at which six of the ten alleles (60%) from five platypuses (three adult females, one juvenile female, one juvenile male) were OranDZB*2. Three copies of OranDZB*59 and one copy of OranDZB*17 were also sequenced from platypuses captured at this location. These findings may suggest that there is a degree of isolation at this site, but further research would be required to confirm this.

The 162-bp sequence found in two platypuses was consistent with a pseudogene. Gene duplication followed by mutation can lead to non-functional sequences of DNA, called pseudogenes, on which selective pressure is not exerted (Bollmer *et al.*, 2010). As a result, mutations or variations in length of a pseudogene are not removed from a population. The length of this sequence being one bp shorter than the DZB gene would

suggest it does not code for a protein. The similarity to the DZB gene would suggest that the two are related, and most likely that the 162bp sequence is a pseudogene (Aguilar *et al.*, 2005). Gene duplication and pseudogene formation is common for classical MHC genes (Axtner and Sommer, 2007; Bollmer *et al.*, 2010), but has not previously been reported in platypuses. While this finding may be of little consequence to platypus health and conservation, further research in this and/or other populations may shed further light on it.

The existence of only a single study for comparison limits the conclusions that can be drawn from this study, and the results can, to a large degree, be considered to supplement the baseline population data reported by Lillie *et al.* (2012), with the important addition that the data of this study can be compared with demographic and individual health data from the same platypuses (Chapter 8). Future research could expand upon the genomic work of this project by including investigation of diversity at additional loci involved with the immune system and loci without immune system function (Acevedo-Whitehouse and Cunningham, 2006). Analysis of microsatellites and mitochondrial DNA could provide further information on gene flow within and between river catchments (Kolomyjec *et al.*, 2009; Gongora *et al.*, 2012; Furlan *et al.*, 2013).

Chapter 5.

Reproductive seasonality

5.1 INTRODUCTION

Knowledge of reproductive physiology and ecology is of great importance in the conservation of wildlife species for a number of reasons. Reproduction is necessary for recruitment of new individuals to replace those that die but it can also drain energy reserves of adults making them more vulnerable to serious health consequences. For example, gestation and lactation can be times of negative energy balance in females (Bronson, 1985; Munks and Green, 1995; Hilderbrand *et al.*, 2000; Cook *et al.*, 2004), and in *Antechinus* spp, elevated corticosteroid levels in males during the breeding season can lead to immunosuppression and death from bacterial or parasitic infection (Barker *et al.*, 1978; Bradley *et al.*, 1980). In addition, the importance of certain habitat factors may vary seasonally according to the timing of breeding and the rearing of young (Chamberlain *et al.*, 1999; Serena *et al.*, 2014). Consequently, understanding the timing of reproduction can be of great importance in interpreting individual health data understanding environmental factors that may affect reproductive success, and assessing population health.

The most striking feature of platypus reproduction, the laying of eggs by the female of a mammalian species, was established in scientific literature in the late 19th century (Caldwell, 1887). Since then, although many details remain to be elucidated, much has been learnt about the physiology and ecology of platypus breeding. Examination of killed specimens in the late nineteenth and early to mid-twentieth centuries provided detailed gross and histological information on the female reproductive system in platypuses from mainland Australia. The female has a left and right ovary; however only the left one is functional and it is only seasonally active (Hill and Gatenby, 1926; Hughes and Carrick, 1978).

The left ovary approximately doubles in size around the breeding season to around 14 x 8 mm in cross-section (Hill and Gatenby, 1926; Temple-Smith, 1973). Several ovarian follicles develop simultaneously, and typically two mature at the same time (Hill and Gatenby, 1926). Mature ovarian follicles form hemispherical protrusions from the ovarian surface, up to 4.75 mm in diameter (Hill and Gatenby, 1926; Hughes and Carrick, 1978). No significant volume of fluid develops within the follicle, which at the time of ovulation has been reported to be filled by the 4-4.5 mm diameter oocyte (Hill and Gatenby, 1926; Hughes and Carrick, 1978). Follicles that fail to mature undergo atresia and their contents are released directly into a unique system of lymphatics within the medullary region of the ovary (Hill and Gatenby, 1926). Follicles that reach maturity form a corpus luteum after ovulation. Corpora lutea project from the surface of the ovary (Hill and Gatenby, 1926). They grow to up to 4 mm across, but regression begins early, when the ovum is in a late cleavage (blastodisc) stage (Hill and Gatenby, 1926).

The ovum develops as it passes through the uterus until it is approximately 17 x 15 x 15 mm in size and contains a blastocyst with a primitive streak several mm in length and a short head process (Hill and Gatenby, 1926). Hawkins and Battaglia (2009) used video monitoring of captive platypuses to estimate gestation to be 15-21 days. A gestation of at least this length is supported by the laying of fertilised eggs by wild females that had been taken into captivity over two weeks previously (Frank Carrick, personal communication).

In the male platypus, there are two functional intra-abdominal testes (Temple-Smith, 1973; Carrick and Hughes, 1978). They are located in the caudal abdomen, caudodorsal

to the caudal pole of each kidney (Temple-Smith, 1973; Carrick and Hughes, 1978). The gross and histological structure of the testis is typical of those of most higher order vertebrates (Temple-Smith, 1973; Carrick and Hughes, 1978). The testes remain inactive during the first year of life (Temple-Smith, 1973; Frank Carrick, personal communication). However, from the second year onwards the testes in males undergo a distinct annual cycle in size and mass (Temple-Smith, 1973).

Most of the information on the timing of seasonal changes in reproductive tract morphology and reproductive hormones in the platypus has been gathered from populations on mainland Australia (Burrell, 1927; Griffiths *et al.*, 1973; Temple-Smith, 1973; Hughes and Carrick, 1978; Griffiths, 1984; Handasyde *et al.*, 1992; Jakubowski *et al.*, 1998; New *et al.*, 1998). Temple-Smith (1973) found that the size of the left ovary and left and right uteri of platypuses in NSW began to increase from June, peaking at about three times the basal size in August/September, and then reduced to a minimum size by December. Jakubowski *et al.* (1998) found similar seasonal changes in plasma progesterone concentrations in female platypuses in the Barnard River Catchment in New South Wales. Monthly mean progesterone values were found to be significantly higher in July-September than in other months. Hughes and Carrick (1978) reported that Burrell (1927) recovered eggs from nesting burrows in NSW between 24th August and 22nd October. Griffiths *et al.* (1973) reported seasonal effects on mammary gland development in platypuses caught in rivers in the Australian Capital Territory that were consistent with a spring breeding season: the mammary glands were found to be quiescent from May until the middle of July, becoming more developed towards the end of July and lactating platypuses were found between the mid-October and April.

Similar evidence of seasonality of breeding is present in male platypuses. Temple-Smith (1973) reported that monthly mean testis size in platypuses from NSW was at a minimum from December to April and peaked in August at around 12 times the minimum value. There was considerable variation in the size of spermatogenic testes between September and December, which was considered to be a result of individual variation or possibly due to age differences (Temple-Smith, 1973). New *et al.* (1998) observed elevated monthly mean androgen levels in male platypuses in NSW in July-September, with the mean value in August being over three times (and significantly) higher than those in all other months including (July and September). Histologically, the testes of NSW platypuses are quiescent in January-March, the initiation of recrudescence of the testis can start in April, and spermatozoa can be produced in small numbers from May onwards (Temple-Smith, 1973). There is high spermatogenic activity in August-October, with regression of the testes in October-December (Temple-Smith, 1973).

These seasonal changes in reproductive tract morphology and reproductive hormones are strong evidence of a breeding season from August-October in mainland platypuses. However, there is evidence that the timing of the breeding season varies with latitude throughout the species' range, occurring later in more southern locations. Peak plasma progesterone concentrations have been observed to occur one month later in Victoria than in NSW (Handasyde *et al.*, 1992; Jakubowski *et al.*, 1998). In addition it has been reported that platypus eggs have been found earlier in Queensland and northern NSW, than in southern NSW and Victoria. Similarly, observations that capture of newly emerged juveniles and lactating females in Tasmanian field studies lags behind that on the mainland by two to three months and observations of testis size in five necropsy

specimens suggest that the breeding season occurs even later in Tasmania (Grant *et al.*, 1983; Grant and Temple-Smith, 1983; Connolly and Obendorf, 1998; Munks *et al.*, 1998; Munks *et al.*, 2000; Bethge *et al.*, 2001; Grant, 2004).

While there is strong evidence for seasonal breeding in the platypus, the endocrine control of platypus reproduction has not been fully determined. From histological examinations, Temple-Smith (1973) characterised platypus spermatogenesis as being prenuptial - starting before the breeding season, continuing during the breeding season then stopping. Despite peak concentrations of androgen for male platypuses from NSW in July-September, spermatogenesis can begin in May (Temple-Smith, 1973). This is likely to be possible because the concentration of androgens in the testes is higher than in the peripheral circulation (Carrick and Hughes, 1978) and is able to support spermatogenesis in some individuals even at the relatively low (but not basal) peripheral concentrations in May (New *et al.*, 1998). The elevated androgen concentrations observed (particularly in August, but also in July and September) may be necessary for development of systemic effects such as full development of accessory reproductive glands, increased libido and territorial aggression (New *et al.*, 1998; Frank Carrick, personal communication). The specific effects of each androgen are not known in platypuses and it is likely that each has different reproductive effects (Frank Carrick, personal communication).

The reason for the elevated progesterone concentrations in female platypuses from NSW in July-September has not been determined. New *et al.* (1998) concluded that the timing of elevated monthly mean progesterone levels suggested a pre-ovulatory surge in progesterone analogous to that in reptiles and birds. However, histological evidence of

the development of a corpus luteum after ovulation and nutrition of the ovum primarily from secretory products from the endometrial lining rather than the ovary suggests that, similar to other mammals, progesterone release during pregnancy is likely (Hill and Gatenby, 1926; Hughes and Carrick, 1978). The latter suggestion is supported by the observations of short-beaked echidnas (*Tachyglossus aculeatus*), one of only four extant species of monotreme other than the platypus) that serum progesterone concentration was highest during gestation (Nicol *et al.*, 2005). There has been little investigation of the role of oestrogens in platypus reproduction, but Hughes and Carrick (1978) found the level of 17β-oestradiol was higher in a full term pregnant platypus than in two non-pregnant individuals and an ovariectomised female.

Breeding success in wild platypus populations has been assessed in live capture studies either on the basis of the proportion of animals captured that are juveniles or by the proportion of females captured during the expected lactation period in which milk let down can be induced by injection of oxytocin (Grant *et al.*, 1983; Connolly and Obendorf, 1998; McLachlan-Troup, 2007; Serena *et al.*, 2014). Ultrasonography has been used to investigate reproductive status in a small number of individuals from two species of monotreme - platypuses and short-beaked echidnas (Oates *et al.*, 2002; Johnson *et al.*, 2007; Nichol and Morrow, 2009; Russell Jones, personal communication). Oates *et al.* (2002) were able to identify the ovaries and uterus on ultrasonography of two anaesthetised short-beaked echidna using a ventral abdominal approach. Morrow and Nicol (2009) captured an ultrasound image of an egg containing a 0.35cm embryo in the uterus of a lightly anaesthetised female short-beaked echidna in dorsal recumbency. Johnston *et al.* (2007) used ultrasonography to measure testicular

volume in seven short-beaked echidnas. Platypus testes have also been imaged, in a captive situation (Russell Jones, personal communication).

The aim of this chapter was to investigate seasonality of reproduction in Tasmanian platypuses, using ultrasonography and serum reproductive hormone concentrations, to allow better interpretation of the demographic, remote monitoring and individual health data gathered as part of the population health assessment framework, and also to investigate the proportion of the individuals examined that were reproductively active. As part of this study, I aimed to develop a technique for performing ultrasonography on the reproductive tracts of male and female platypuses, to describe the ultrasonographic appearance of the surrounding structures, and make observations of seasonal changes in reproductive organs.

5.2 METHODS

5.2.1 Study animals

A total of 154 individual platypuses (63 adult females, 3 juvenile females, 76 adult males, 6 subadult males, and 6 juvenile males) were captured, with 12 recaptures, in the Inglis Catchment in northwest Tasmania between 29/8/11 and 31/8/13 (Chapter 2).

5.2.2 Ultrasonography of internal reproductive organs

Abdominal ultrasonography was used as a method of assessing the internal reproductive organs of 61 platypuses (31 adult males, one subadult male, four juvenile males, 23 adult females, two juvenile females), on one occasion each, between 16/9/12 and 26/2/13.

Ultrasonography was performed under isoflurane anaesthesia with platypuses in dorsal recumbency. Previous work has found that, in unclipped platypuses, 10-20 minutes were required to massage ultrasound gel into thick fur to allow adequate contact for abdominal ultrasonography (Russell Jones, personal communication). Therefore, to minimise the duration of anaesthesia, in this study a 4 x 2cm area of fur was clipped at ~30° from sagittal on the left side of the ventral aspect of the abdomen, between the ribs and the epipubic bone (Figure 5.1). To minimise the effects of clipping on thermoregulation after release, this was only performed for captures between the spring and summer months of September and February. MES Conductive gel (Medical equipment services, Melbourne, Victoria) was applied to the skin in the clipped area to ensure good contact of the transducer with the skin (Figure 5.1). An Aloka Prosound 2® ultrasound unit (Hitachi Medical Corporation, Tokyo, Japan) with a 4cm linear transducer was used, at 10Mhz. Images described as being in the sagittal plane or transverse planes were likely to be at angles of up to the 30° to these planes.

The landmarks for locating the left testis and left ovary were the right ventral process of the spleen and the left kidney. The platypus spleen has a left dorsal process and a right ventral process (Mackenzie, 1916; Connolly *et al*., 1999) which was used for orientation. Measurements from images were taken using the ultrasound unit, or the Foxit Phantom pdf distance measure tool (Foxit Software Incorporated, Fremont, CA, USA), which can measure the distance between any two points on an image after the scale from a known measurement on that image is set (Table 5.1).

Table 5.1 Measurements recorded during abdominal ultrasonography in the platypus (*Ornithorhynchus anatinus*) (in cm).

Measurement		Orientation
Left kidney	length	sagittal
	height	sagittal
	width	transverse
	height	transverse
Left kidney to spleen distance		sagittal
Ventral splenic lobe	width	sagittal
	height	sagittal
Splenic blood vessel	width	sagittal
	height	sagittal
Males only:		
Left kidney to left testis distance		sagittal
Left testis	length	sagittal
	height	sagittal
	width	transverse
	height	transverse
Females only:		
Left kidney to left ovary distance		sagittal
Left ovary	length	sagittal
	height	sagittal
	width	transverse
	height	transverse
Left ovarian follicle	width	sagittal
	height	sagittal
	width	transverse
	height	transverse

Sagittal plane testis cross-sectional areas were calculated using the formula:

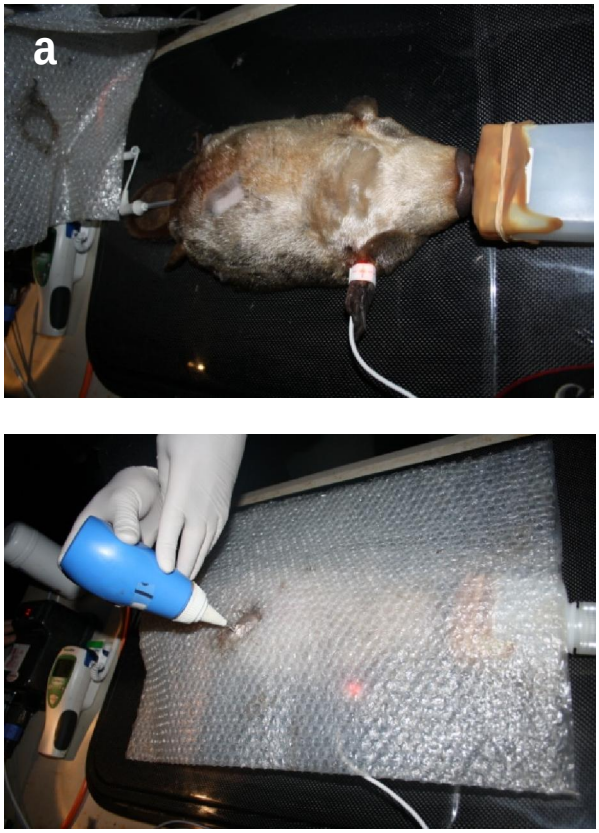
Cross-sectional area (cm^2) = $(\pi/4)$ * length (cm) * height (cm)

Testis volumes were calculated using the formula:

Volume (cm^3) = $(1\pi/6)$ * sagittal length (cm) * ((sagittal height + transverse height (cm))/2) * transverse width (cm)

Total body length (tbl) of each individual was measured. Testis area and volume results were standardised against tbl^2 and tbl^3, respectively.

Figure 5.1. Preparations for ultrasonography: a) window of clipped fur, and b) application of ultrasound gel through hole in bubble wrap blanket to minimise heat loss. **Photos: Geoff Dutton.**



5.2.3 Endocrinology

Blood samples were collected under anaesthesia from the venous bill sinus of wild platypuses, as described in Chapter 7, between 11/9/12 and 28/8/13. Samples were centrifuged and serum separated and then stored at -20°C. From the sera that were of sufficient volume for endocrinology after biochemistry and serology (see Chapter 7) samples were selected to give a relatively even distribution of animals tested across the 12 months of the year. Progesterone and 17β-oestradiol were assayed in serum from one capture of n=31 female platypuses (n=29 adults and n=2 juveniles) or two captures of n=2 females (adult Platypuses 95 and 107). Testosterone was assayed using serum from

one capture of n=35 male platypuses (n=29 adults, n=4 subadults and n=2 juveniles), one capture of n=2 females and two captures of n=1 female (Platypus 95).

Extraction of bound hormones was not performed so the assay results represent free serum hormone levels. Progesterone was assayed using the Correlate- EIA Progesterone Enzyme Immunoassay Kit (Enzo Life Sciences, Inc., Farmingdale, New York). Testosterone was assayed using the ENZO Testosterone ELISA kit (Enzo Life Sciences, Inc., Farmingdale, New York). Oestradiol was assayed using the ENZO Life Sciences 17β-Estradiol ELISA kit. These kits are competitive enzyme immunoassays. Each test involves competitive binding of sample hormone and a known concentration of enzyme linked hormone to a polyclonal antibody to the respective hormone followed by washing of the hormone bound antibodies and the addition of the enzyme substrate. The enzyme/substrate reaction causes a colour change in the solution, the degree of which can be measured by optical density at a specific light frequency and used to determine the sample hormone concentration. Each sample was assayed in duplicate by Dr Geoff Dutton at Charles Sturt University using the kit protocols.

5.2.4 Detection of sperm on microscopic examination of material on cloacal swabs

During examination, cloacal swabs of male platypuses were collected for laboratory tests including microscopy (Chapter 7). Spiral structures 30-50µm in length, seen on microscopy of material from cloacal swabs of male platypuses, were initially considered to be consistent with spiral bacteria. However, due to the shape and size of the structures, their seasonal detection and the fact that they were only detected in samples from male platypuses, they were later determined to be sperm. The dates on which sperm were detected were recorded.

5.2.5 Statistical analysis

The significance of seasonal variations in testis size and hormone levels were tested using the Mann Whitney U test using Statistica 8.0 (Stat Soft Inc. Tulsa OK, USA).

5.3 RESULTS

5.3.1 Left abdominal ultrasonographic landmarks

The right ventral process of the spleen was imaged in cross section in the sagittal plane, as a homogenous, hypoechoic, half tear drop shaped structure adjacent to the ventral body wall in 56 platypuses (Figure 5.2 a). It was usually located directly under the clipped area of skin. The left kidney appeared as an oval structure with mixed echogenicity in both sagittal and transverse planes (Figure 5.2 b & c). It was always located more dorsally than the right ventral process of the spleen (Figure 5.2 b). It was at least partly cranial to the spleen in 53 of these platypuses (Figure 5.2 b), directly dorsal to the spleen in two platypuses and at least partly caudal to the kidney in six platypuses. A small oval or circular anechoic area adjacent to the craniodorsal margin of the right ventral process of the spleen was imaged in 23 platypuses (Figure 5.2 d). Rotation of the probe demonstrated this to be a cylindrical rather than a spheroidal structure (Figure 5.2e), consistent with a blood vessel adjacent to the surface of the spleen. On two occasions it was noted that there was a smaller anechoic oval area adjacent to the above mentioned one, likely an artery and vein in close proximity (Figure 5.2 f). In 17 platypuses, the left dorsal process of the spleen was imaged as a long, homogenous, hypoechoic structure running cranio-caudally adjacent to the dorsal body wall (Figure 5.2 g). Because of the position of the clipped area, the urinary bladder was imaged as an anechoic structure in the caudal abdomen, only in five individuals (two adult males, two adult females, one juvenile male) (Figure 5.2 h). The left testis

was imaged as a homogenous oval structure of intermediate echogenicity in both sagittal and transverse planes (Figure 5.3). It was located caudal or caudoventral to the left kidney, and caudal or caudodorsal to the right ventral process of the spleen. It was possible to image the testis and kidney in the same 4cm wide field of view in six platypuses (Figure 5.2 i). The ovaries that were imaged in two females (see Section 5.3.3) were located in a similar position to the testis in the male (Figures 5.6 and 5.7). The dimensions of selected structures around the internal reproductive organs of the platypuses from which appropriate images were captured are listed in Table 5.2.

Figure 5.2. Ultrasound images of selected structures around the internal reproductive organs: a) right ventral process of spleen in sagittal plane, dotted lines indicate height and length, b) sagittal view of kidney, dotted lines indicate height and length, c) left kidney, dotted lines indicate height and width, in transverse plane, d) right ventral process of spleen and associated blood vessel, labelled "BV" with dorsal and ventral margin each indicated by +, in sagittal plane, e) right ventral process of spleen and associated blood vessel, labelled "BV" with dorsal and ventral margin each indicated by +, in transverse plane, and f) right ventral process of spleen and associated blood vessel, labelled "BV", in sagittal plane.

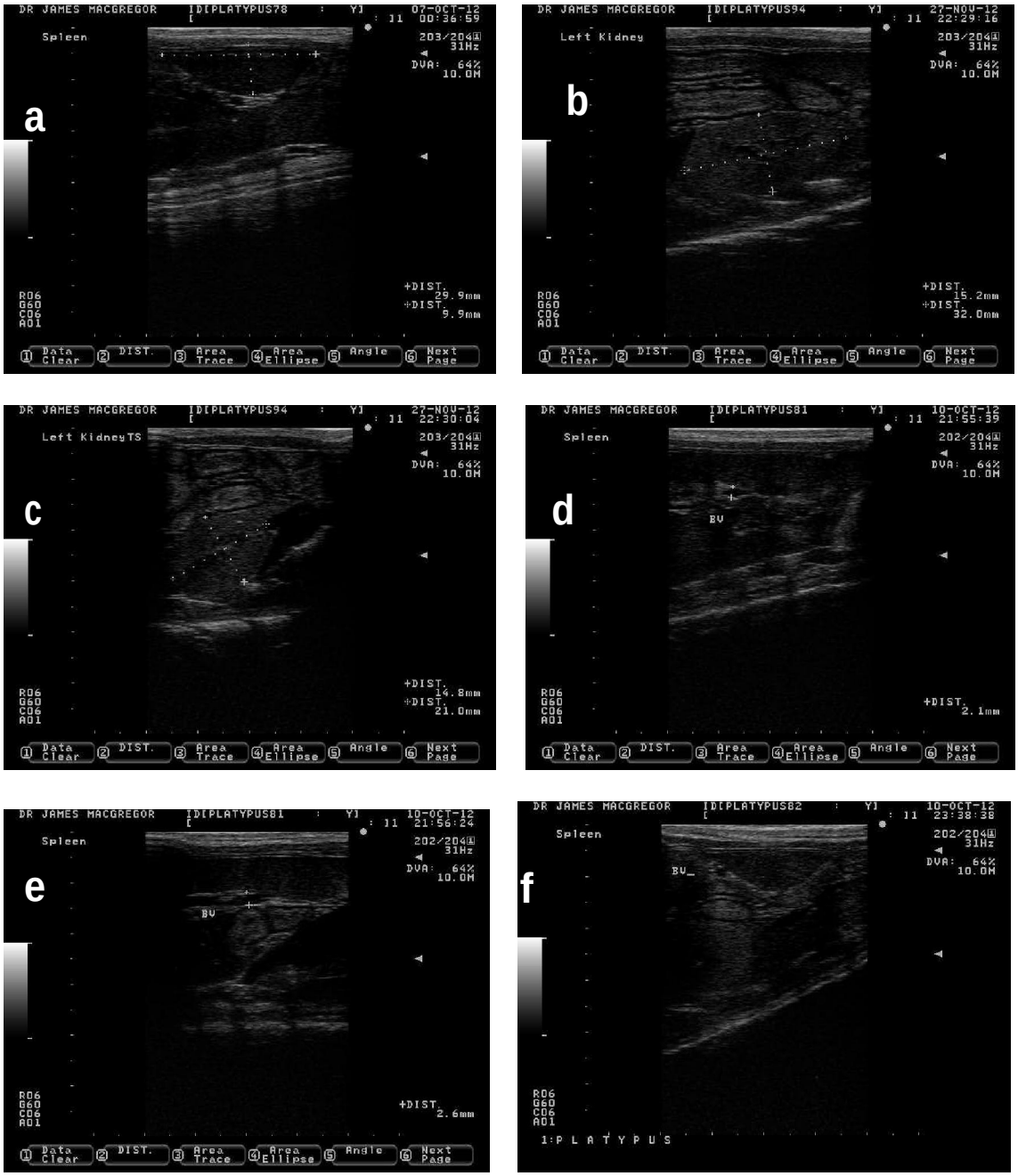
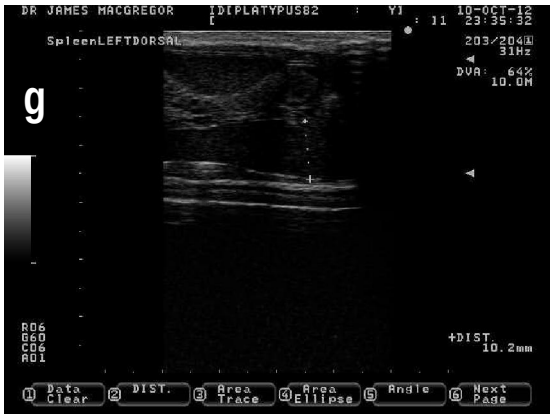


Figure 5.2 (Cont'd). Ultrasound images of selected structures around the internal reproductive organs: g) left dorsal process of spleen in tranverse plane, dotted line indicates height, h) sagittal view of urinary bladder in sagittal plane, dotted line indicates height, and i) single image showing kidney (K), spleen (S) and testis (T) in sagittal plane.



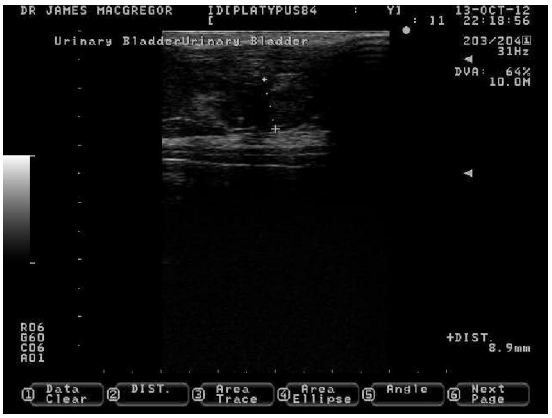


Table 5.2. The sizes dimension (cm) of selected structures around the internal reproductive organs. Sag = sagittal plane. Trans = transverse plane

			Female			Male		
			Mean	**SD**	**n**	**Mean**	**SD**	**n**
Left kidney	**length**	Sag	2.82	0.33	22	3.15	0.34	29
	height	Sag	1.53	0.12	23	1.71	0.23	30
	width	Trans	2.45	0.05	2	2.43	0.32	11
	height	Trans	1.41	0.08	2	1.66	0.17	11
Left kidney - ventral process of spleen distance		Sag	0.38	0.25	20	0.61	0.26	26
Left kidney - left testis dist.		Sag				1.13	0.83	6
Left kidney - left ovary dist.		Sag	1.18		1			
Ventral process of spleen	**width**	Sag	2.42	0.38	21	2.56	0.37	26
	height	Sag	0.82	0.14	23	0.83	0.09	28
Dorsal process of spleen	**height**	Sag	0.88	0.18	10	0.97	0.20	6
Bv adjacent to vent. spleen	**width**	Sag	0.26	0.07	10	0.24	0.08	11
	height	Sag	0.17	0.04	10	0.17	0.04	10

5.3.2 Reproductive ultrasound of male platypuses

The left testis was imaged in 35 platypuses (31 adult, 1 subadult, 3 juvenile). Images of a large testis (Platypus 93; Figure 5.3 a & b) and a small testis (Platypus 120; Figure 5.3 c & d) are shown below. The dimensions of the left testis are summarised in Table 5.3.

Figure 5.3. Ultrasound images of left testis in two adult platypuses: a) Platypus 93 in sagittal plane, b) Platypus 93 in transverse plane, c) Platypus 120 in sagittal plane, and d) Platypus 120 in transverse plane.

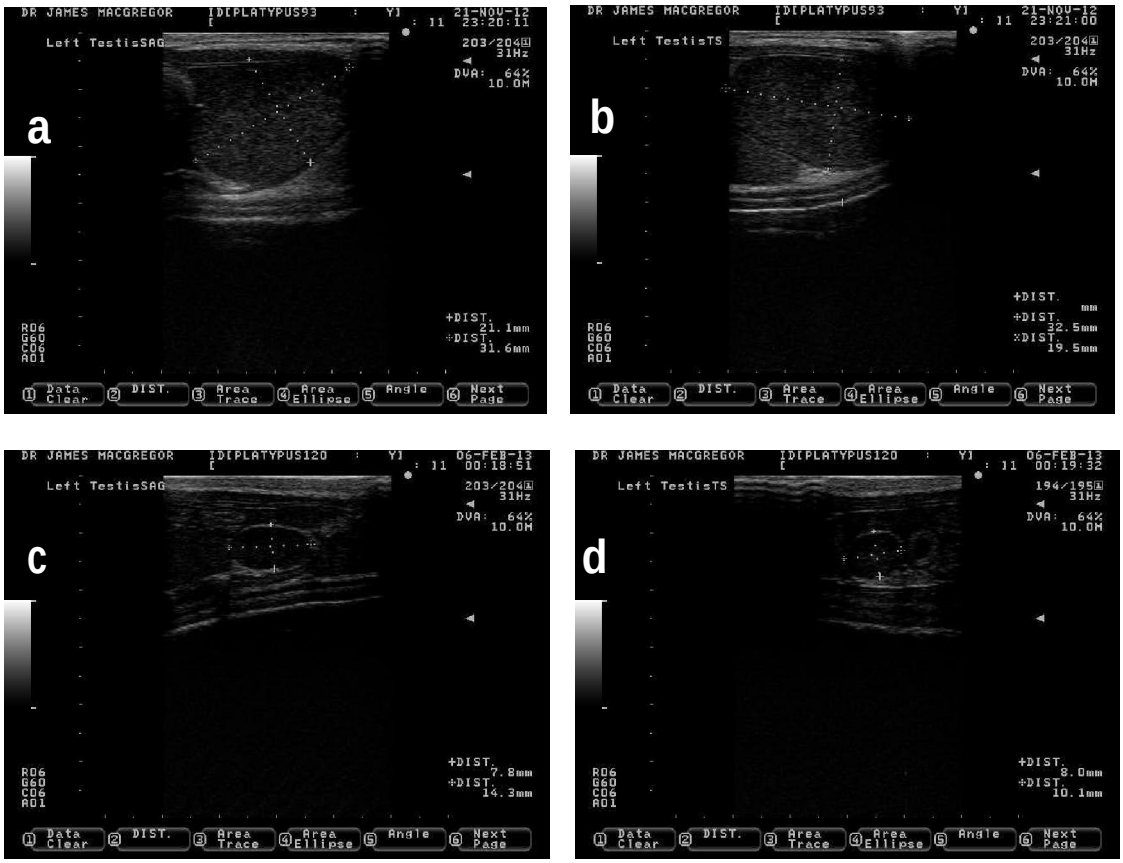


Table 5.3. Left testis dimensions (cm). sag = sagittal plane, trans = transverse plane

	Adult			Subadult			Juvenile		
	Mean	**SD**	**n**	**Mean**	**SD**	**n**	**Mean**	**SD**	**n**
Length (sag)	2.43	0.76	31	0.45	N/A	1	1.17	0.30	3
Height (sag)	1.48	0.47	31	0.39	N/A	1	0.74	0.17	3
Width (trans)	2.21	0.61	23	0.8	N/A	1	-	-	-
Height (trans)	1.47	0.44	24	0.44	N/A	1	-	-	-

Seasonal values for parameters based on testis cross-sectional area in the sagittal plane and testis volume are illustrated in Figures 5.4 and 5.5, respectively.

Figure 5.4. Seasonal values for a) calculated testis cross-sectional area in sagittal plane, and b) calculated testis cross-sectional area in sagittal plane/total body length^2 in the platypus (*Ornithorhynchus anatinus*) in the Inglis Catchment, Tasmania.

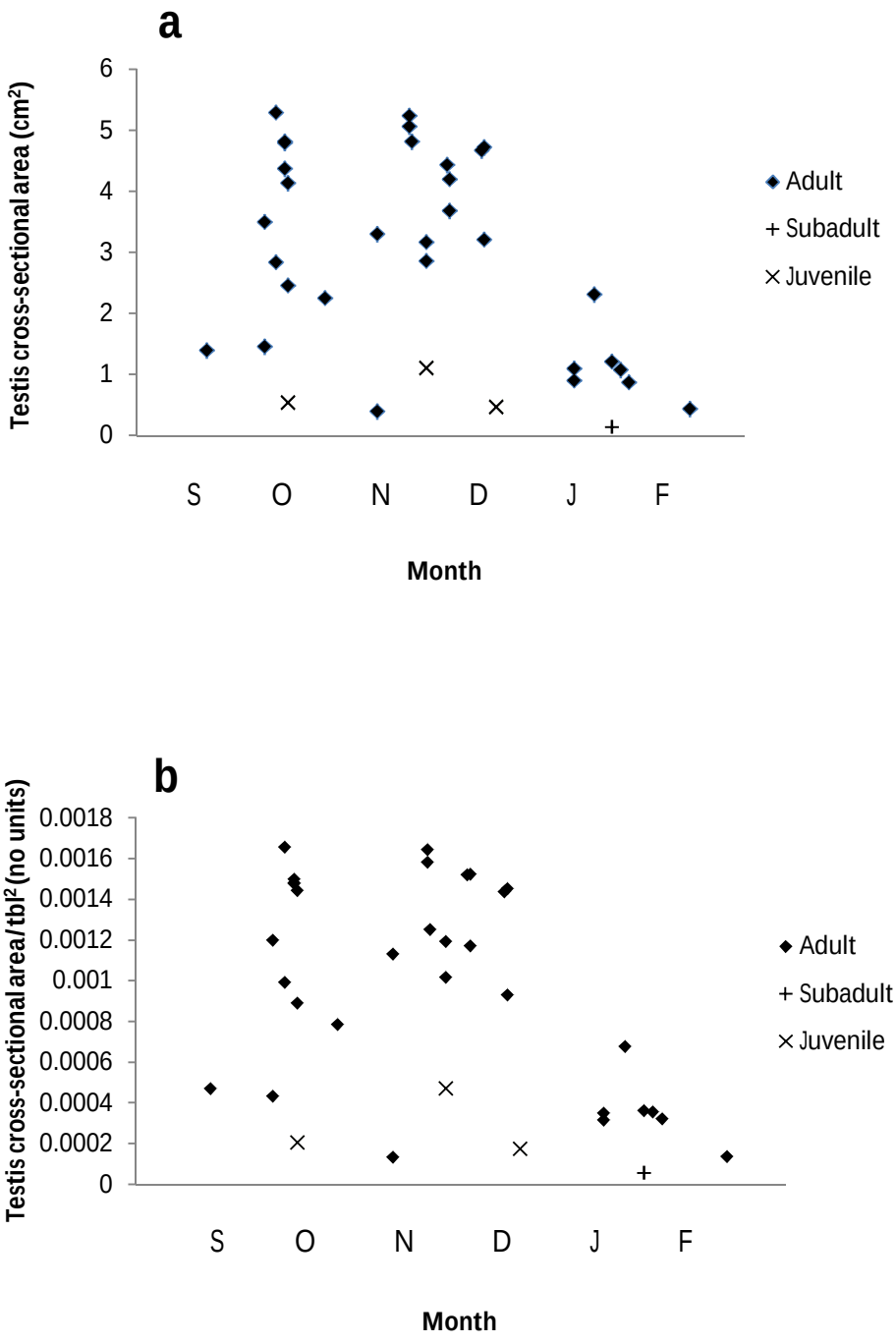


Figure 5.5. Seasonal values for a) calculated testis volume, b) calculated testis volume/total body length^3, and c) calculated testis volume/body mass.

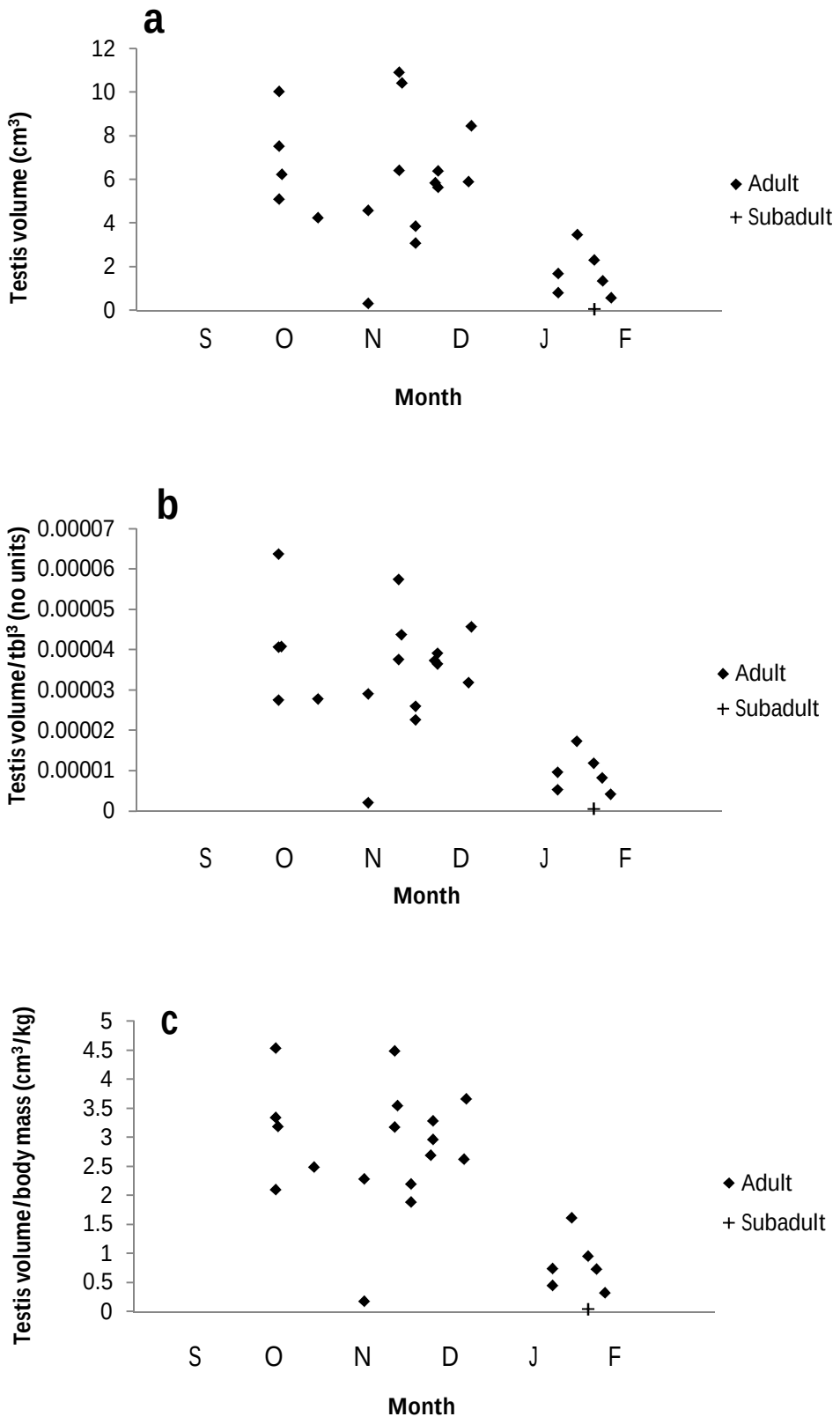


Table 5.4 shows that Mann Whitney U tests found the distributions of all testis area and testis volume based parameters to be significantly different between September-December and January-February. Platypus testes were found to be significantly larger in the months September to December when compared with those from January to February, regardless of the method of measurement (cross-sectional area or volume or testis measurement/body measurement).

Table 5.4. Results of Mann Whitney U tests for testis parameter values in October-December versus January-February.

	Median (Sept – Dec)	Median (Jan-Feb)	U	n (Sept-Dec)	n (Jan – Feb)	p
Testis cross-sectional area in sagittal plane	3.91	1.08	10.00	24	7	**<0.001**
Testis cross-sectional area in sagittal plane/tbl^2	0.00123	0.00035	9.00	24	7	**<0.001**
Testis volume	5.91	1.53	7.00	17	6	**0.002**
Testis volume/tbl^3	0.0000373	0.0000090	6.00	17	6	**0.002**
Testis volume/body mass	2.97	0.74	3.15	17	6	**0.001**

5.3.3 Reproductive ultrasound of female platypuses

An ovoid structure consistent with an ovary was imaged caudal to the left kidney and the right ventral process of the spleen in two (Platypuses 95 and 107) of the 23 adult female platypuses examined by abdominal ultrasonography. These two individuals were captured in November and December 2012 and appeared to be in good health (Figure 5.6 and 5.7). The largest cross-sectional dimensions imaged for these structures were 1.08x1.07cm and 1.13x1.65cm in Platypuses 95 and 107, respectively. In both cases the oval structure was of moderate echogenicity and contained a relatively large anechoic structure. In Platypus 107, a second distinct anechoic structure was imaged in

the transverse plane, smaller than and ventral to the large anechoic structure (Figure 5.7 c - e). In Platypus 95, there was a less distinct and even smaller hypoechoic area ventral to the large anechoic area in the sagittal plane. The appearances of the anechoic and hypoechoic structures were consistent with those of ovarian follicles.

Figure 5.6 (a = sagittal plane). Structures consistent with ovary and ovarian follicle in Platypus 95, dimensions indicated by dotted lines where present.

Figure 5.6. (Cont'd; b = sagittal plane, c = transverse plane). Structures consistent with ovary and ovarian follicle in Platypus 95, dimensions indicated by dotted lines where present.

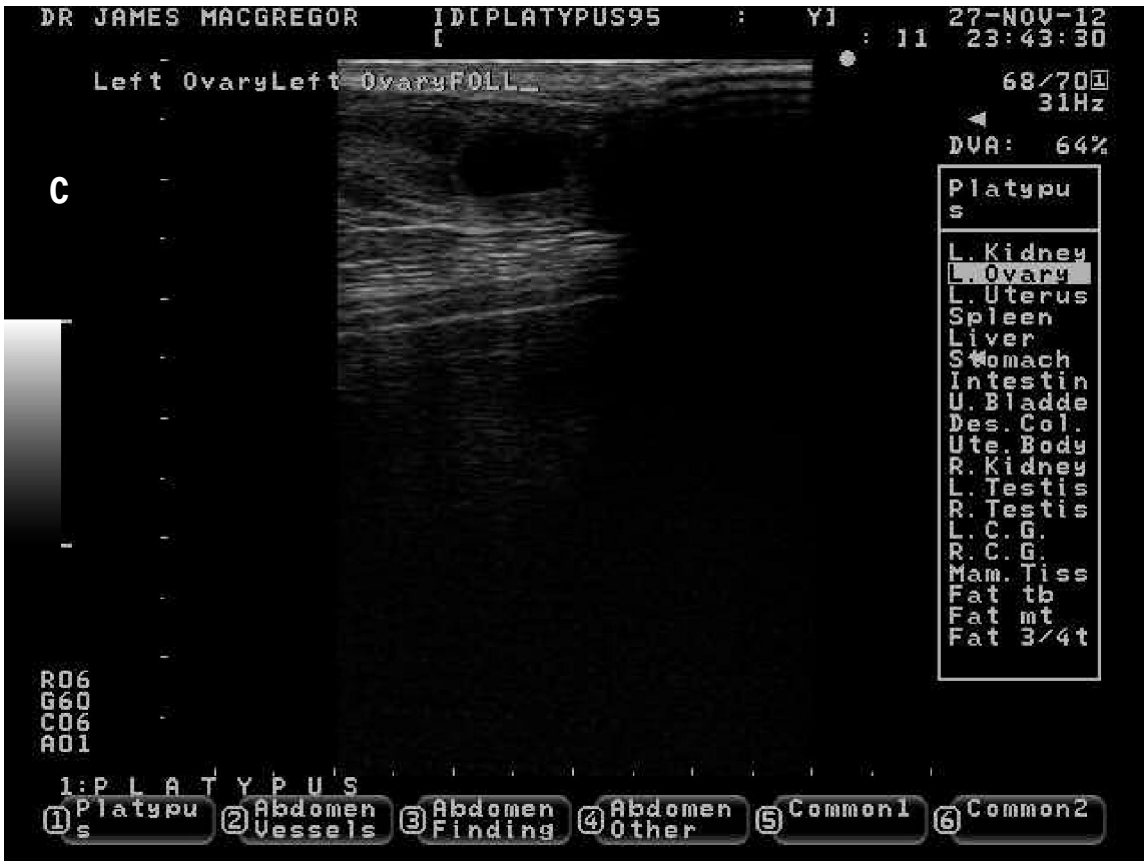


Figure 5.6. (Cont'd; d = transverse plane). Structures consistent with ovary and ovarian follicle in Platypus 95, dimensions indicated by dotted lines where present.

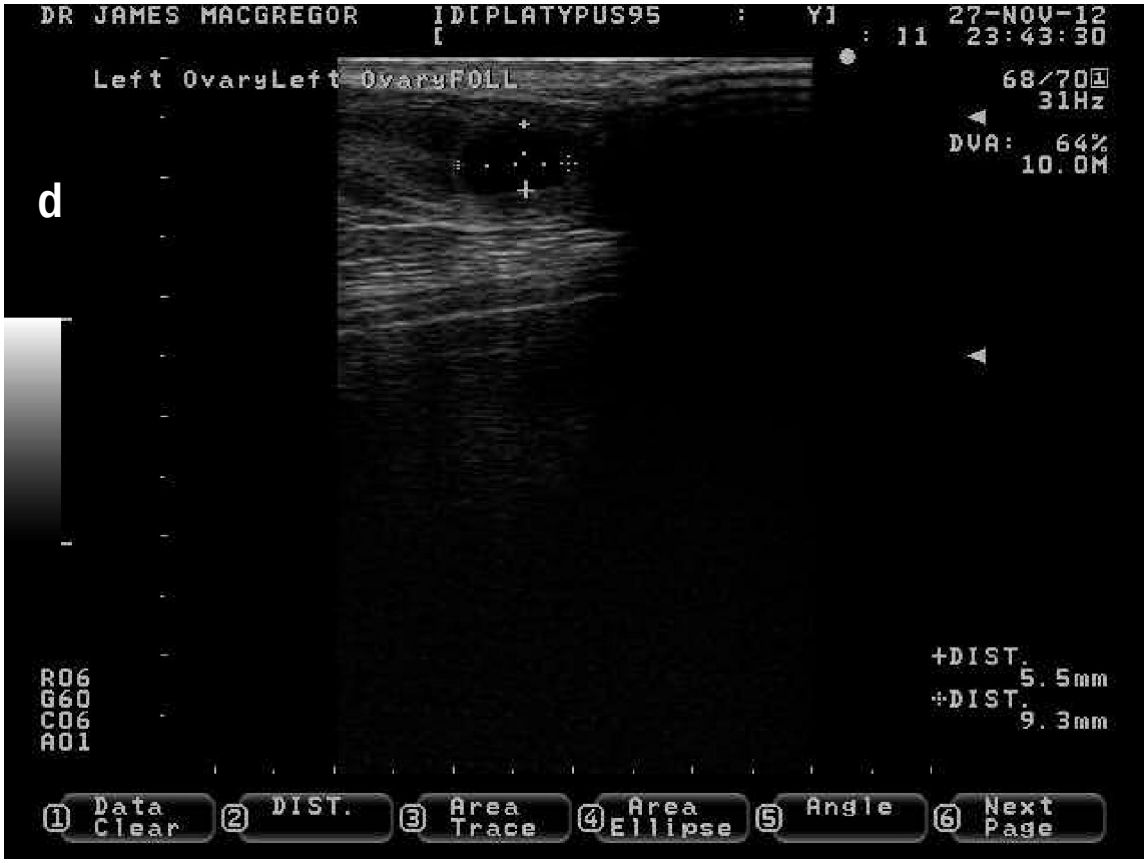


Figure 5.7 (a and b = sagittal plane,). Structures consistent with ovary and ovarian follicles in Platypus 107, dimensions indicated by dotted lines where present.

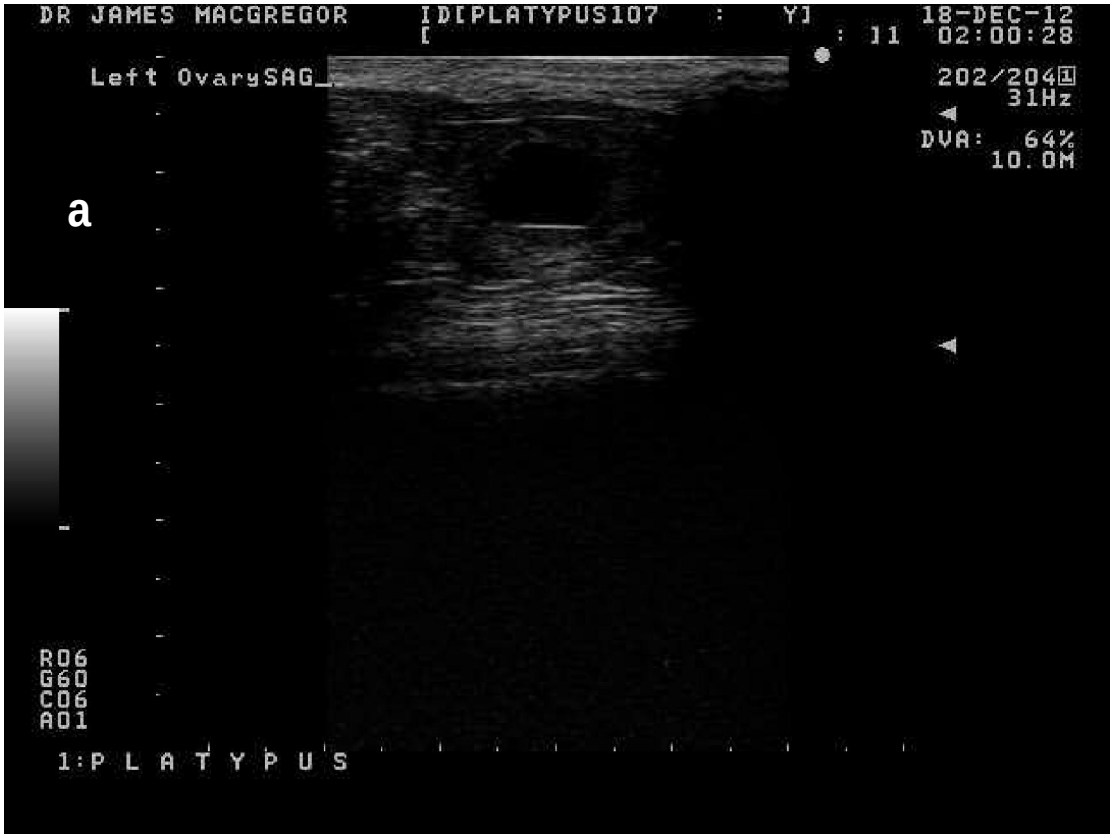


Figure 5.7 (Cont'd; c = sagittal plane, d = transverse plane). Structures consistent with ovary and ovarian follicles in Platypus 107, dimensions indicated by dotted lines where present)

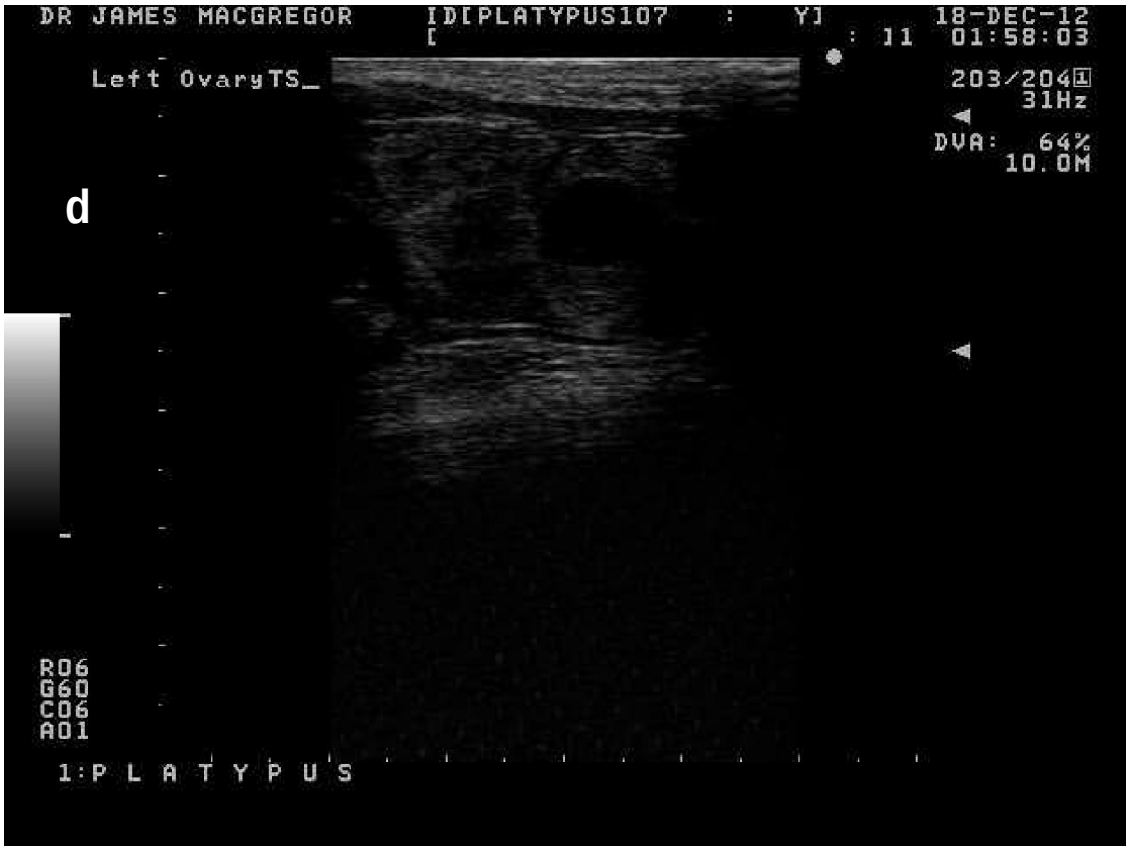
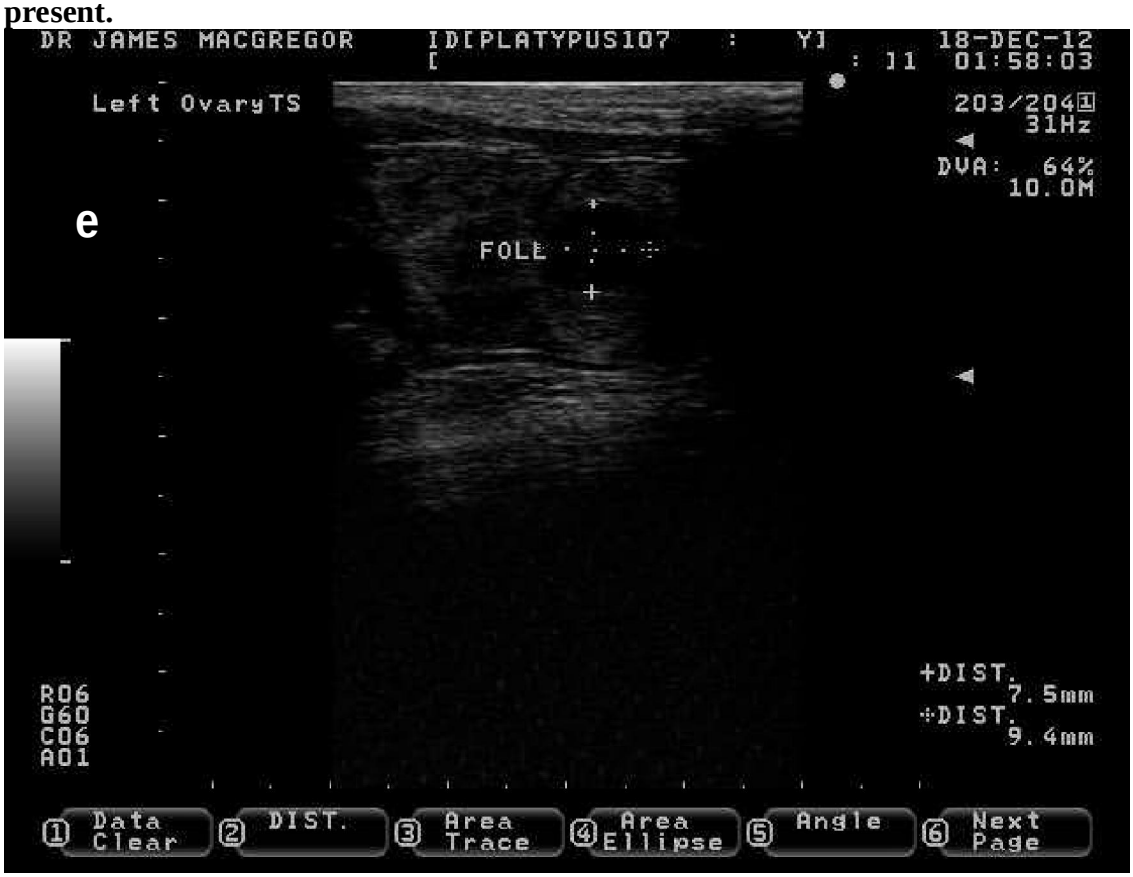
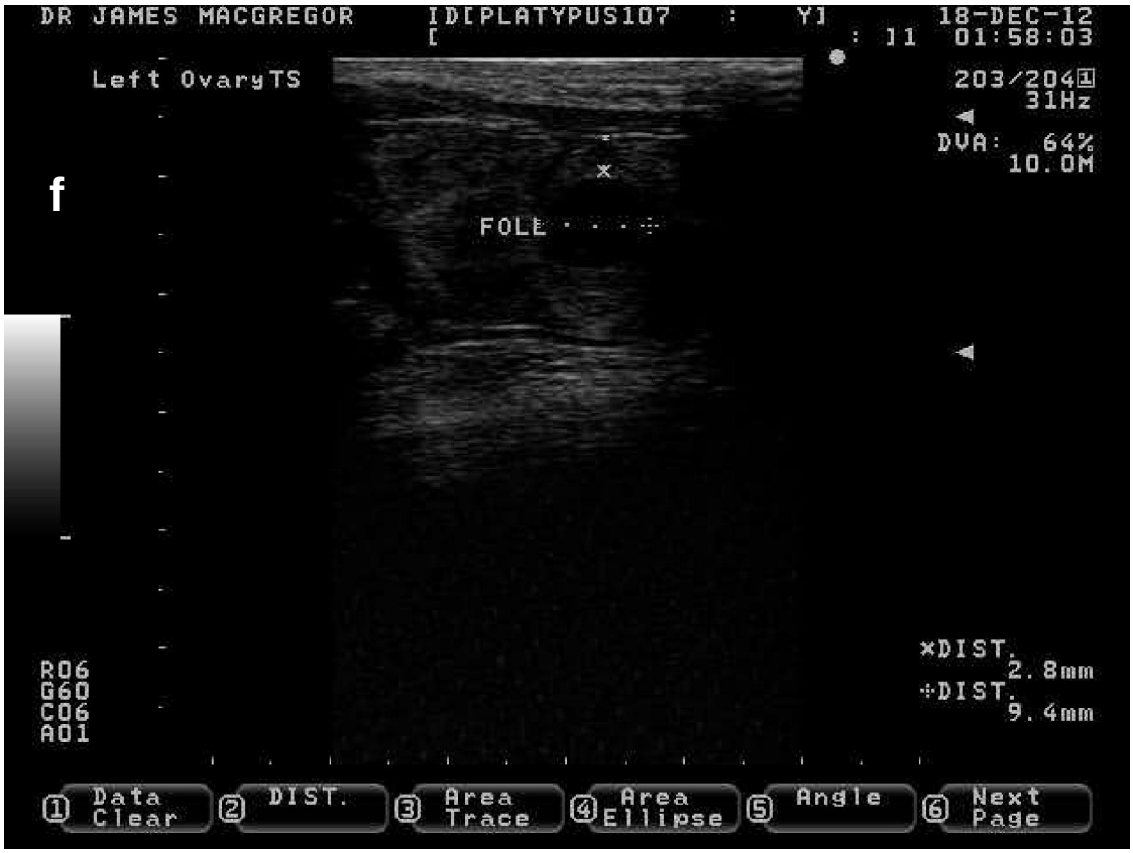


Figure 5.7 (Cont'd; e and f = transverse plane). Structures consistent with ovary and ovarian follicles in Platypus 107, dimensions indicated by dotted lines where present.

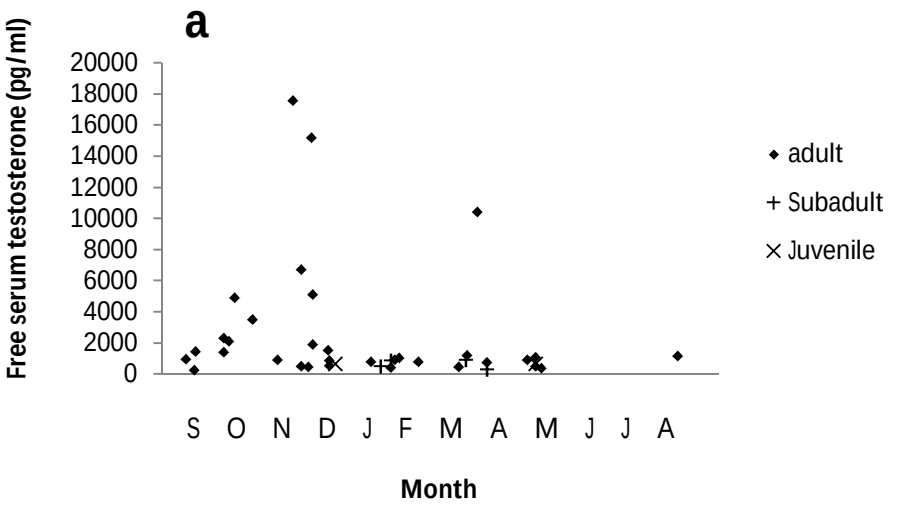


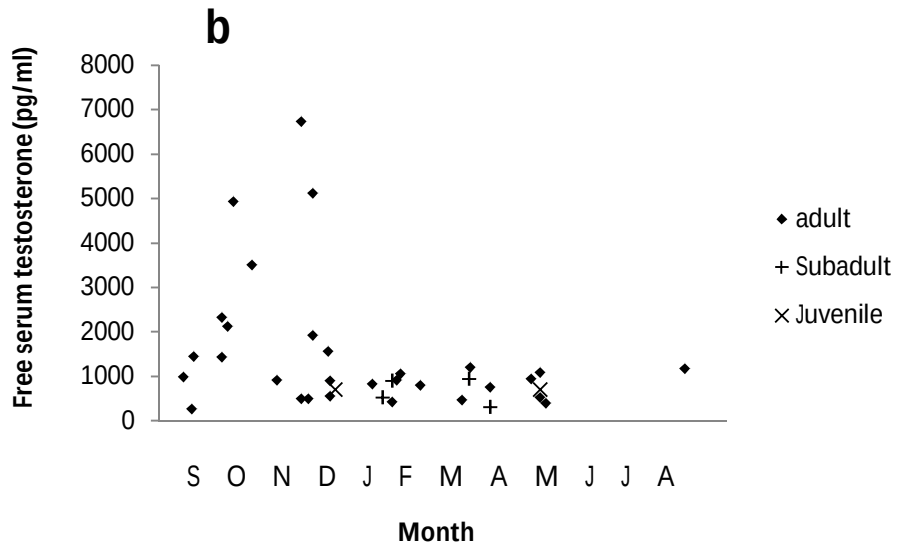


5.3.4 Reproductive endocrinology

Figure 5.8 shows free serum testosterone for male platypuses plotted against date as well as testis volume/tbl^3. Figure 5.9 shows free serum progesterone, 17β-oestradiol and testosterone for female platypuses plotted against date, and Figure 5.10 shows free serum progesterone plotted against free serum 17β-oestradiol.

Figure 5.8. Male hormone data: a) Free serum testosterone vs date, b) Free serum testosterone (excluding three outlier results) versus date, and c) Free serum testosterone (excluding three outlier results) versus testis volume/tbl^3.





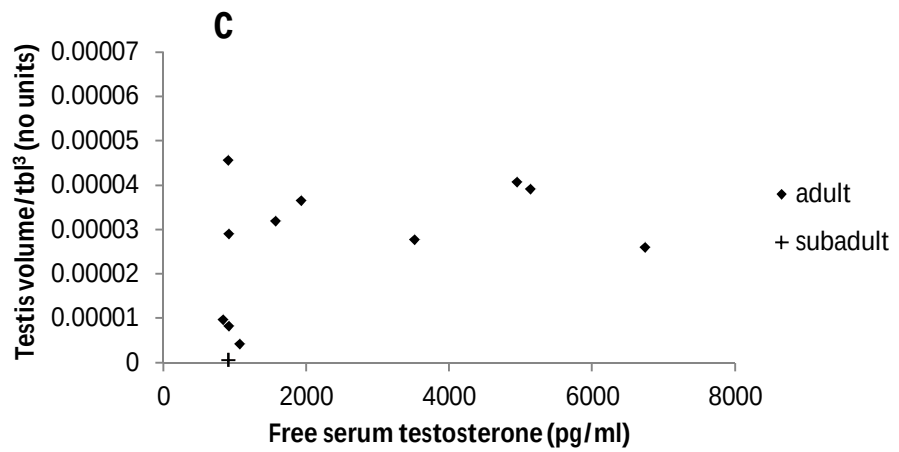
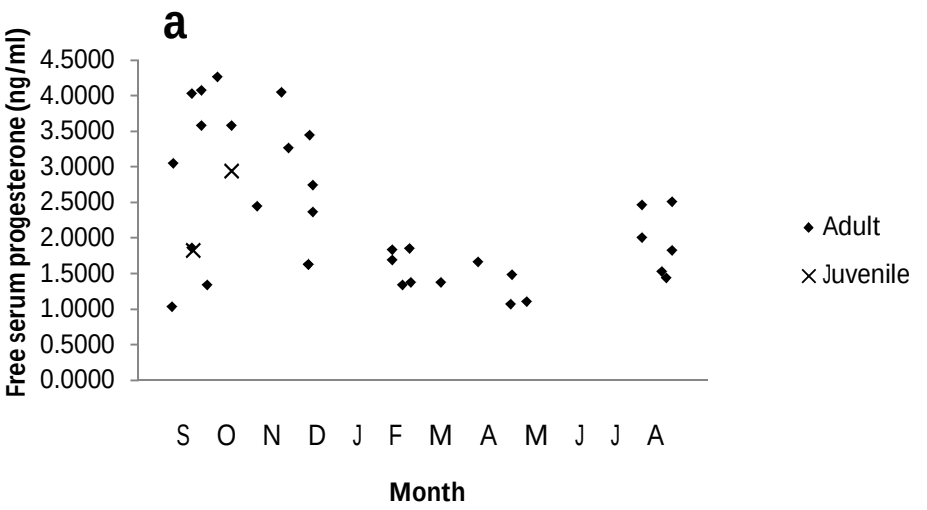
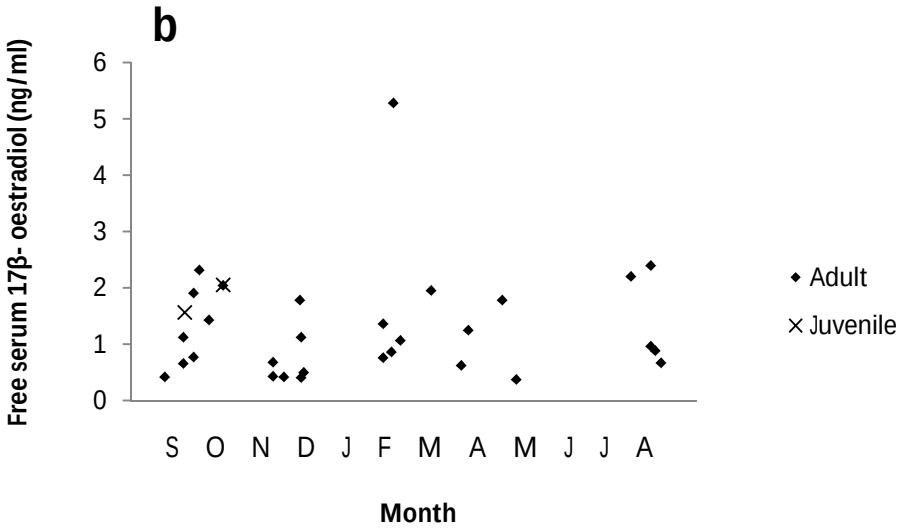


Figure 5.9. Female hormone data: a) Free serum progesterone versus date, b) free serum 17β-oestradiol versus date, and c) free serum testosterone versus date.





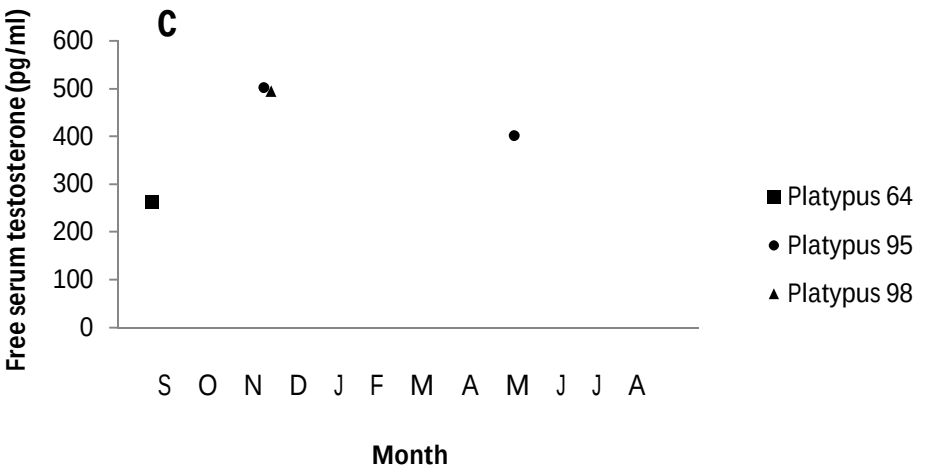
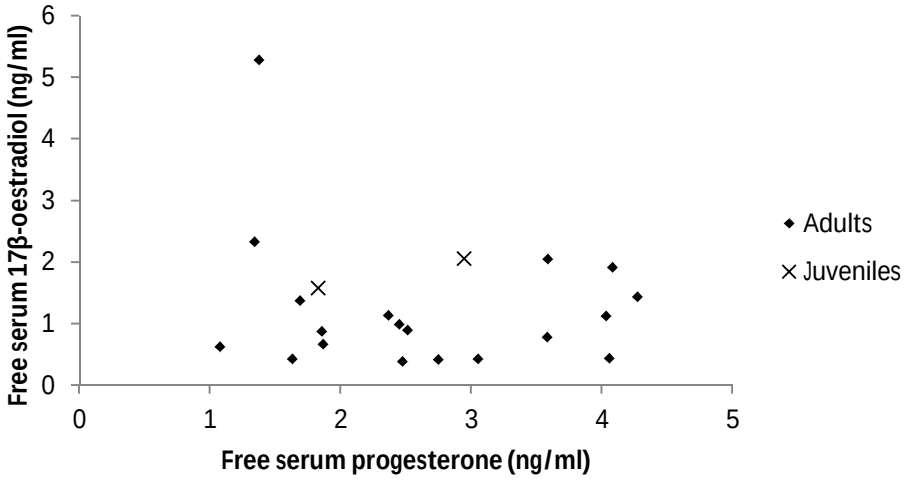


Figure 5.10. Female free serum progesterone versus free serum 17β-oestradiol



For four male platypuses, testis volume/tbl^3 was at least 2.5 times smaller than those of all ten of the other male platypuses with results for both parameters. Although there was no significant correlation between serum testosterone concentration and testis volume/tbl^3 (r^2=0.43, p=0.l68), a Mann Whitney U test showed that serum testosterone concentrations for the four platypuses with the smallest testes (median =908.9) and those of the other platypuses (median = 2,719.1) were significantly different (U=4, p=0.048).

In female platypuses, there was no apparent seasonal pattern in free serum 17β-oestradiol concentrations (Figure 5.9) and no apparent correlation between free serum 17β-oestradiol and free serum progesterone concentrations (Figure 5.10). The two platypuses that were captured and sampled twice (Platypuses 95 and 107) both had ultrasonographic findings consistent with the presence of a large ovarian follicle at their first examination. Progesterone and 17β-oestradiol concentrations were lower at the

second capture in these females (Table 5.5). Free serum testosterone was also slightly lower in Platypus 95 at her second capture Figure 5.9.

Table 5.5. Free serum hormone concentrations on each capture of two female platypuses that were assayed twice. Large ovarian follicle present at first capture of each platypus.

	Platypus 95	Platypus 107
Date 1	27/11/2012	17/12/2012
Progesterone 1 (ng/ml)	4.05	3.44
17β-oestradiol 2 (ng/ml)	0.44	1.13
Date 2	18/05/2013	28/08/2013
Progesterone 2 (ng/ml)	1.11	2.51
17β-oestradiol 2 (ng/ml)	<0.42	0.67

The three highest male testosterone results were considered to be outliers due to the episodic release of testosterone (Section 5.4) and were not included in statistical analysis. Because Platypus 107 had results from two captures in the same month category (August-December), the hormone concentrations from her second capture were not included in statistical analysis. The 17β-oestradiol result of Platypus 95 was below the lower limit of the test read range (0.42ng/ml) and could not be included in Figure 5.9 or in statistical analyses. The 17β-oestradiol result from one platypus was excluded because the results of the paired assays for that sample differed by a factor of ten, suggesting one was not diluted as required by the test protocol. Mann Whitney U tests found the distributions of free serum testosterone concentrations in males and free serum progesterone concentrations in females to be significantly different between August-December and January-July (Table 5.6).

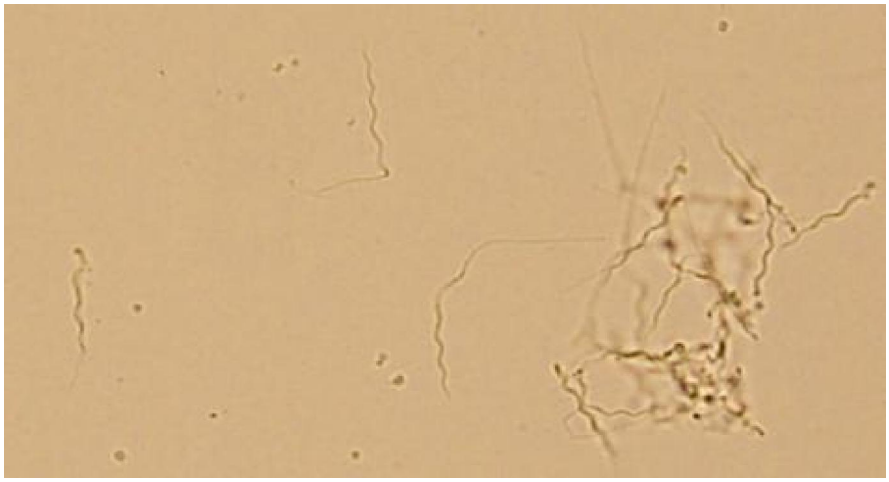
Table 5.6. Results of Mann Whitney U tests for free serum testosterone concentrations in males and free serum 17β-oestradiol and progesterone concentrations in females in August-December versus January-July.

	Median (Aug – Dec)	Median (Jan-July)	U	n (Aug-Dec	n (Jan - July	p
Male testosterone	1560	815	21.00	15	12	**<0.001**
Female progesterone	2.47	1.43	37.00	23	10	**0.002**
Female 17β-oestradiol	0.94	1.26	73	22	9	0.258

5.3.5 Detection of sperm on microscopic examination of material on cloacal swabs

Sperm (30-50µm in length; Figure 5.11) were seen on microscopy of material on cloacal swabs in eight adult male platypuses (n=7 at a single capture, n=1 at two captures). This consisted of 3/10 (30%) adult male captures in October, 4/11 (36%) in November, 1/7 (14%) in December and 1/8 (13%) in January. Sperm were not detected in any other months (n=45). Platypus 9 was captured on three occasions – November 2011, May 2012 and November 2012. Sperm were detected on cloacal swab samples from this platypus in both November 2011 and November 2012, but not in May 2012.

Figure 5.11. Image of platypus sperm as seen on microscopy of material from cloacal swabs a male platypuses. **Photo: Graeme Knowles.**



5.4 DISCUSSION

Seasonal changes in testis volume, male serum testosterone concentrations and female serum progesterone concentrations observed in this study provide strong evidence that platypuses are seasonal breeders and, consistent with the findings of other Tasmanian studies, that the breeding season in platypuses in the Inglis Catchment occurs two to three months after that in mainland platypuses (Table 5.7). While the seasonal patterns in reproductive hormones and testis volumes in this study were very similar to those observed in studies of mainland platypuses, the peak values were found in October-December rather than August and September (Temple-Smith, 1973; Handasyde *et al.*, 1992; Jakubowski *et al.*, 1998; New *et al.*, 1998). Similarly, sperm were only found on microscopy of cloacal swabs between October-January, and ovarian follicles were only found on ultrasonography in November and December.

Table 5.7. Comparison of evidence for seasonality in reproduction in platypus on mainland Australia and Tasmania.

Reference	Method	J	F	M	A	M	J	J	A	S	O	N	D
Australian mainland													
Temple-Smith (1973)	Ovary/uterus size						↑	↑	↑↑	↑↑	↑	↑	
Temple-Smith (1973)	Testis size					↑	↑	↑	↑↑	↑	↑	↑	
Jakubowski *et al.* (1998)	Plasma progesterone conc.							↑	↑	↑			
New *et al.* (1998)	Androgen levels							↑	↑↑	↑			
Temple-Smith (1973)	Spermatozoa production				↑	↑	↑	↑	↑↑	↑↑	↑↑	↑	↑
Burrell (1927)	Eggs recovered from nests								↑	↑	↑		
Griffiths *et al.* (1973)	Mammary gland development	↑↑	↑↑	↑↑	↑↑			↑	↑	↑	↑↑	↑↑	↑↑
Williams *et al.* (2013)	Newly-emerged juveniles	↑	↑↑	↑↑	↑↑	↑	↑	↑	↑				
Tasmania													
This study	Testis volume										↑↑	↑↑	↑↑
Connolly and Obendorf (1998)	Testis size											↑↑	
This study	Serum progesterone conc.								↑	↑↑	↑↑	↑↑	↑↑
This study	Serum testosterone conc.										↑↑	↑↑	↑↑
This study	Spermatozoa production	↑									↑	↑	↑
This study	Ovarian follicles											↑	↑
Connolly and Obendorf (1998)	Lactating females		↑↑	↑↑		↑↑						↑↑	↑↑
Connolly and Obendorf (1998)	Newly-emerged juveniles			↑	↑	↑	↑	↑	↑				
Munks et al. 2000	Newly-emerged juveniles				↑	↑	↑						

ˈ some evidence, ↑ evidence, ↑↑ peak concentration/size/development.

While the pattern of seasonal change in hormones was similar to that found in other studies, it should be noted that the hormone concentrations observed in this study were considerably lower than those in previous studies (Handasyde *et al.*, 1992; Jakubowski *et al.*, 1998; New *et al.*, 1998). There has also been variation in magnitude between the results of previous studies, likely associated with different methods of hormone analysis (Handasyde *et al.*, 1992; Jakubowski *et al.*, 1998). The likely reason for most of the variation between this study and previous studies is that previous studies performed hormone extraction before hormone level analysis whereas this study did not (Handasyde *et al.*, 1992; Jakubowski *et al.*, 1998). As a result, it was free serum hormone concentrations that were measured in this study as opposed to total hormone concentrations in previous studies. In a range of species, testosterone, progesterone and oestrogen are found in blood either weakly bound to albumin, strongly bound to

hormone binding globulins (primarily sex hormone binding globulin (SHBG) for testosterone and oestrogens and cortisol binding globulin (CBG) for progesterone) or in a free, unbound state (Elliott *et al.*, 2003). A specific progesterone binding protein has been described in pregnant guinea pigs (Graham and Clarke, 1997). In healthy men, total testosterone levels consist of free hormone (~2%), albumin bound hormone (~50%), SHBG bound hormone (44%) and CBG bound hormone (~3.5%) (De Ronde *et al.*, 2006). In healthy women, ~2% of total progesterone is in the free state (Al-Asmakh, 2007). The degree to which these different forms of the hormone are bioavailable has not been fully determined, but the sum free and albumin bound hormone concentrations is frequently referred to as the bioavailable hormone level (Winters *et al.*, 1998; Elliott *et al.*, 2003; Tsai *et al.*, 2004). However, different studies in humans have found different relationships between free, bioavailable and total testosterone concentrations (Winters *et al.*, 1998). Total testosterone concentration is strongly correlated with SHBG and concentrations of this protein have been found to vary, seasonally or otherwise, in humans and wildlife (Audy *et al.*, 1982; Winters *et al.*, 1998; Elliott *et al.*, 2003; Suresh *et al.*, 2008). Although it is not possible to measure bioavailable progesterone, CBG has also been found to vary seasonally in the bird species, *Junco hyemalis* (Deviche et al., 2001). It is likely that bioavailable hormone concentrations - not free hormone concentrations as used in this study, nor total hormone concentrations as used in previous studies - are the most appropriate parameters to measure when assessing reproductive function (Winters *et al.*, 1998; Elliott *et al.*, 2003; Tsai *et al.*, 2004). However, despite this, the similar seasonal patterns between different assay methods in this and previous studies suggest that a real effect is being observed.

The considerably higher free serum testosterone values observed for three platypuses in this study were likely to be a result of episodic release of testosterone which has been observed to be a feature of testosterone production in a range of species (Moor and Younglai, 1975; Muduuli *et al.*, 1979; Maurel *et al.*, 1981; Pelletier *et al.*, 1982; Cooke *et al.*, 1993). In humans, episodic release has been shown to lead to greater fluctuations in free testosterone concentrations than total testosterone concentrations (Cooke *et al.*, 1993). This could result in greater fluctuations in the testosterone concentrations found in this study compared to previous studies and to the observation of these three outlier values. Pelletier *et al.* (1982) and Muduuli *et al.* (1979) observed that episodic peaks in testosterone concentrations were more frequent but only of slightly greater magnitude during the breeding season of sheep and goats, respectively. Such a finding is consistent with the frequency and magnitude of high free testosterone results in this study.

The endocrine control of reproduction, and the source of reproductive hormones, in female platypuses have not been established. Jakubowski *et al.* (1998) suggested that because progesterone levels appear to be elevated before the breeding season, progesterone may be released before ovulation in the platypus as it is in some reptiles. Hill and Gatenby (1926) suggested that in platypuses, corpora lutea serve an endocrine function but reported that they began to regress several days before egg-laying. Hughes and Carrick (1978) supported the suggestion that the platypus corpus luteum releases progesterone. In echidnas, Nicol *et al.* (2005) found progesterone levels to be highest 1-2 days before egg laying and questioned whether either preovulatory progesterone release or early corpus luteum regression could be a feature of monotreme reproduction. At this stage, there is not enough data to determine which, if any, of the above are correct. For instance, although the corpus luteum is a common source of progesterone in

a range of species, there are other sources. The placenta is one example but is not present in the platypus (Tuckey, 2005). However, the adrenal gland produces progesterone in some species and is a possible source in the platypus (Fajer *et al.*, 1971; Plotka *et al.*, 1983; Frank Carrick, personal communication). A short peak in oestrogen release is a common finding prior to ovulation in mammalian species (Döcke and Dörner, 1965). In this study, the free serum 17β-oestradiol result for one platypus captured in February was at least twice as high as each of the results for other platypuses. It is possible that in this platypus ovulation was occurring later than the main breeding season as suggested by the other results. There is evidence from a captive situation that more than one oestrus may occur in a breeding season in individual females that do not become pregnant at their first oestrus (Hawkins and Battaglia, 2009). Alternatively, the elevated 17β-oestradiol result observed may have been an aberrant result. The suggestion by Jakubowski *et al.* (1998) that female reproduction in platypuses is similar to that in some reptiles led to four assays of female serum testosterone being performed in this investigation. Testosterone has been shown to be elevated in reproductively active females in some reptile species (Lovern and Wade, 2001). Although based on only four samples from three individuals, the higher female serum testosterone concentrations found in the breeding season in this investigation may indicate that a full assessment of the endocrine control of female platypus reproduction should involve assessment of testosterone. Such an investigation would most likely only be possible in a captive situation where repeat samples can be taken from the same individual(s).

The range of calculated testis volumes in this study was similar to the range of those calculated from direct measurement in necropsy specimens by Temple-Smith (1973).

However, the mean body mass of adult males captured by Temple-Smith was 1.46 ± 0.31k (n=106), but the mean body mass of adult males captured in this study was 2.06 ± 0.31kg (n=76; Chapter 7). The distributions of male body mass results in these two studies is statistically significant ($t=12.8$, $p<0.001$). Direct comparison of testis volume in relation to body size between the two studies is not possible because Temple-Smith (1973) used the parameter testis mass/body mass which cannot be determined from ultrasound data.

One adult male platypus had a testis volume that was much lower than others during the breeding season. It is possible that this platypus, captured in November, was incorrectly classified as an adult. However, Williams *et al.* (2013) found that juveniles captured in Victoria retained at least part of their spur sheaths and would be identifiable as non-adults until at least November of their first year after emergence. The earliest time for complete loss of spur sheaths would be expected to be later in Tasmania on the basis of the findings of this study. Alternatively, it is possible that this was a two year old platypus that was late in reaching sexual maturity. This platypus otherwise appeared to be in good health. If it was greater than two years old, there may have been undetected health problems that lead to small testis volume when compared to other adult males at the same time of year.

The ovarian follicles observed in this study differ from previous reports of platypus ovarian follicles in that their maximum diameters were approximately twice as large as reports from necropsy specimens (Hill and Gatenby, 1926; Hughes and Carrick, 1978). This could result from a combination of two factors. Firstly, the necropsy specimens were mainland platypuses that are generally smaller than Tasmanian platypuses

(Connolly and Obendorf, 1998) and secondly necropsy measurements appear to have been taken from the ovarian surface rather than from within the ovary where the follicle diameter may have been greater (Hill and Gatenby, 1926; Hughes and Carrick, 1978).

One of the aims of this investigation was to use ultrasonographic evidence of ovarian activity as an indicator of the number of reproductively active females. However, structures consistent with ovaries were found in only two female platypuses. This low number is thought to be an artefact of the method used and not a true indication of the number of females that are reproductively active in the population. This is the first study that has attempted to find platypus ovaries by ultrasonography, so there were no existing methodologies that could be followed, and the left platypus ovary could be difficult to locate for a relatively inexperienced operator. In addition, due to the year round nature of the fieldwork schedule only eight female platypuses were captured in the two months when ovarian follicles were observed, and even during these months reproductively active female platypuses may only have had well developed reproductive structures for a proportion of the time. Confirmation of the timing of the breeding season in this study suggests that any future attempts to use ultrasonography of the female reproductive tract to assess the percentage of females that are reproductively active in these study populations, should focus fieldwork efforts on the months of October, November and December.

Two other measures have been commonly used in previous studies in the evaluation of reproductive success at different stages of the reproductive cycle - the proportion of juveniles captured and the proportion of females that are found to be lactating (Grant *et al.*, 1983; Connolly and Obendorf, 1998; McLachlan-Troup, 2007). The major

disadvantage of the former is that juveniles have been observed to migrate over 40km (Serena and Williams, 2013) and juvenile capture rates do not distinguish juveniles bred in an area from those that have migrated into it. There are also potential issues with the safety and specificity of observations of milk let down after oxytocin injection during the months when the females are most likely to be lactating (McLachlan-Troup, 2007). With regards to safety, oxytocin causes myometrial contraction in eutherian mammals and can lead to early oviposition in birds (Rząsa and Ewy, 1970; Giraldi *et al.*, 1990). As a result it would be preferable not to inject oxytocin into a platypus that has a developing egg within its uterus. However, waiting too long after the anticipated main breeding season may lead to a high level of false negative results. While it is likely that this technique is at least as good a method of detecting ovarian activity as reproductive ultrasound, it does not necessarily imply the female is suckling young. The mammary glands have been shown to enlarge and develop in all adult female platypuses during the breeding season (Griffiths *et al.*, 1973), and while it has been assumed that those that go on to produce milk are suckling young, the latter has not been explicitly demonstrated. False pregnancy and spontaneous lactation occurs in a range of species (Creel *et al.*, 1991) and until more is known about platypus reproduction, it cannot be excluded as a possibility in the platypus (Frank Carrick, personal communication). Demonstration of developing eggs within the uterus by ultrasonography may provide better evidence of reproduction than either ultrasonography of the ovary or milk let down after oxytocin, or at least add to information gathered by these techniques. Further work with abdominal ultrasonography based on the findings of this study, and with fieldwork concentrated during the expected breeding season, could determine if this is likely to be a useful technique. Interestingly, the remote monitoring section of the population health assessment project, which was designed primarily to monitor survivorship and

migration, unexpectedly showed potential as a non-invasive method of monitoring reproductive activity at suitable locations (Chapter 3).

Confirmation of the timing of the breeding season in Tasmanian platypuses through the results of this study is important for understanding the health and behaviour of individuals, assessment of the health of populations and more broadly for the development of conservation management plans for the species. Serena *et al.* (2014) has shown evidence of varying effects on reproductive success of rainfall levels at different times of year and concluded that this was likely to be a result of effects on food availability for adults during the breeding season and the vulnerability of juveniles to flooding before they emerge from their burrows at 3-4 months of age. I have also been made aware anecdotally of sightings of dead juvenile platypuses during/shortly after a flooding event in the Inglis Catchment in January 2011. It seems likely that human activities around water bodies (such as dam building or dredging, riparian vegetation management, or bridge building) may affect food availability for adult platypuses and/or the safety of juveniles in their burrows if they are undertaken during the breeding season or when juveniles are confined to their burrows. The current Tasmanian Platypus Management Plan (Gust and Griffiths, 2010) recommends avoiding "major earthworks near waterways from December to April each year when adult female platypuses and their young will be in their breeding burrows". The findings of this study would suggest this recommendation should be extended to include November at least. More generally this study will improve the ability of all Tasmanian land managers to give consideration to the reproductive seasonality when determining the risk to platypuses of human activities in and around water bodies.

Chapter 6.

Comparison of techniques for assessing body condition

6.1 INTRODUCTION

Body condition is a measure of an animal's energy reserves (Green, 2001). Assessment of body condition is therefore an important aspect of the clinical examination of any animal, although methods of making this assessment vary between species and the reliability of some methods has been questioned (Green, 2001; Schulte-Hostedde *et al.*, 2005). Development of objective methods which can standardise body condition assessment are therefore of value.

The current generally accepted method of assessing platypus body condition is the Tail Volume Index (TVI), which is based on the appearance of the tail and the degree and ease of ventral folding of the edges of the tail at its midpoint when gentle pressure is applied (Grant and Carrick, 1978). TVI is scored from 1 to 5 with lower values indicating higher tail volume (Grant and Carrick, 1978). Two studies provide the basis for the use of the TVI. Firstly, Temple-Smith (1973) described that on post-mortem examination the tail was the primary deposit of fat in the platypus. He also observed parallels between seasonal changes in body mass and two indices of tail volume. The first of these was the relative tail volume which was defined as the tail volume measured by water displacement/tail length. The second was the Tail Fat Index (TFI) which was defined as the cross-sectional area of the tail at its mid-point determined by external measurement/tail length. Secondly, in ten dead platypuses Hulbert and Grant (1983) found that the tail contained a mean of 43.1±3.5% of the total body fat and found no other localization of fat. They used an injection of tritiated water and subsequent blood sampling to demonstrate an association between total body fat and relative tail volume, they also showed parallels between seasonal changes in body mass, tail volume

and body fat, and that the relative mean tail volumes for platypuses in each TVI category increased as TVI decreased.

The use of TVI to measure platypus body condition is very simple and convenient and can be completed in a few seconds. As a result, all published studies that have assessed body condition subsequent to those above have done so using TVI as described by Grant and Carrick (1978), with the exception of Serena and Williams (1997) who amended the description of the categories described by Grant and Carrick (1978) and added two extra categories (3+ between 2 and 3, and 3- between 3 and 4). However, TVI is largely a subjective measure and (in my experience) it is not uncommon to be in doubt as to which of two adjacent categories to place a platypus. The effects of this could be compounded by the fact that most platypuses end up with a score of 2, 3 or 4. Less importantly, the platypus TVI scale places animals of greater body condition in a lower TVI category and vice versa, which is inconsistent with condition scores systems in other species.

In addition to these limitations of using TVI, four aspects from the studies by Temple-Smith (1973) and Hulbert and Grant (1983) suggest that further investigation is warranted to validate the use of TVI as a reliable method of assessing platypus body condition:

- In ten post mortem specimens, Hulbert and Grant (1983) found that the proportion of body fat that was in the tail ranged from 30.4 to 59.9%. Data was not presented to relate this variation to the amount of body fat or tail fat present. This variation may be a source of considerable inaccuracy in the use of TVI as a body condition index.

- Temple-Smith (1973, p52) stated that the deposits of fat, other than that in the tail, "…appeared to be used in preference to the tail fat reserves. Often, individuals with reasonably large tail fat reserves had little or no remaining subcutaneous fat reserves". This suggests that a platypus may show little or no change in tail fat volume while losing up to half of its body fat from other areas.
- Despite considerable overlap in the total body water (negatively correlated with body fat) between September and February, Hulbert and Grant (1983) found no overlap in the relative tail volumes between these two months. This could suggest a different distribution of fat within the body at different times of year. One possible reason could be redistribution of fat away from the tail to insulate the rest of the body at colder times of the year.
- Additionally, while the similar patterns of weight and tail volume indices found in Temple-Smith (1973) and Hulbert and Grant (1983) provide compelling evidence that on average the parameters change together in their study populations, there was no data presented on whether they correlate in individuals.

It has been common for ecologists to produce body condition indices for wildlife based on body mass and body size and there has been a considerable amount of discussion published in the last 20 years about the relative merits of different methods of body condition index calculation (Jakob *et al.*, 1996; Viggers *et al.*, 1998; Green, 2001; Schulte-Hostedde *et al.*, 2005). Simple measures of the ratio between body mass and a linear measure of body size (LM) have largely been discounted (Jakob *et al.*, 1996; Green, 2001; Schulte-Hostedde *et al.*, 2005). Multiple linear regression of body mass against more than one LM has also been recommended, although Green (2001)

described problems with the assumptions of using this technique. Use of the residuals of an Ordinary Least Squares (OLS) linear regression of body mass against a transformed or untransformed LM has been the most commonly used method in recent years (Peig and Green, 2010), however OLS attributes all deviation of data points from the regression line to error in one of the two variables whilst assuming that there is no measurement error in the other. In the case of body mass and an LM, there is likely to be measurement error in both. For example practical difficulties and operator inconsistency may lead to error in LM measurement; and these same reasons as well as the variable quantity of ingesta or waste products within an animal's body at the time of measurement may lead to error in body mass measurement. When creating a body condition index, it is therefore difficult to choose which variable should be considered to have no measurement error, and a different line of best fit will be produced depending on which is chosen.

Reduced Major Axis (RMA) regression similarly produces a linear relationship between sets of data for two variables. However, unlike OLS, RMA regression takes into account measurement error in both the variables. Reduced Major Axis regression will produce a linear relationship that is different to either of the possible outcomes from OLS. Although there has been debate about whether RMA regression is more appropriate than OLS regression for use in the development of body condition indices, there appears to be consensus that when there is measurement error in both body mass and LM, RMA regression is more appropriate.

A range of data transformations have been used in body condition indices, including log transformations of body mass and LM, or a power transformation of LM, but often with

little justification of which transformation was chosen (Green, 2001). Use of LM^3 has been common but LM^2 has also been used. If it can be assumed that individuals of different size within a species have body dimensions in the same proportions, then for individuals of the same body condition, body mass is related to LM^3. However, a uniform shape for individuals of different sizes should not be assumed (Green, 2001). The theoretical basis of determining a power relationship between body mass and body size by regression of logarithm (body mass) against logarithm (LM) (i.e. log-log plots or ln-ln plots) has been recognised for decades (LeCren 1951) and is consistent with a mathematical technique that has been in use for much longer. However, use of this technique to develop condition indices with a degree of validation has historically been rare. Peig and Green (2009 and 2010) reported that the log-log approach using RMA regression performed better than six other methods of determining body condition.

The aim of this study was to investigate the reliability of platypus body condition indices to assist the interpretation of individual health data in the population health assessment framework. To do this, I intended to develop novel measures of body condition, and to investigate the relationships between these and previously reported measures of body condition, in order to make recommendations for body condition assessment in this and future platypus research projects.

6.2 GLOSSARY OF TERMS AND ABBREVIATIONS

BCI 1	Body condition index 1; body mass/ total body length^3
BCI 2	Body condition index 2; body mass/ bill width$^{3.2}$
Bill length	Length of bill without shield (mm)
Bill width	Width of bill at its widest point (mm)

LM	Linear measure of body size
Mid-tail fat depth	Dorso-ventral thickness of fat adjacent to the bone and musculature of tail at the mid-point along the tail's length
Mid-tail fat area	Cross-sectional area of the fat in the tail at the mid-point along the tail's length
RFD 1	Relative Fat Depth 1; 10^6*mid-tail fat depth$^{1.7}$/ total body length^3
RFD 2	Relative Fat Depth; 10^6*mid-tail fat depth$^{1.7}$/ bill width$^{3.2}$
RTFV 1	Relative Tail Fat Volume 1; 10^4*tail fat volume/ total body length^3
RTFV 2	Relative Tail Fat Volume 2; 10^4*tail fat volume/ bill width$^{3.2}$
RMA	Reduce Major Axis regression
Tail length	Distance from the tip of the tail (not including length of hair cover) to the level of the most caudal part of the body (mm).
TFI	Tail Fat Index - Cross-sectional area of the tail at its mid-point (determined by measuring height and width and assuming the area has the shape of an arc of a circle) divided by tail length.
Total body length	Length from tip of bill to tip of tail (cm)
TVI	Tail Volume Index - determined by assessing the degree to which the lateral edges of the tail can be folded downwards with gentle downward. Higher values indicate poorer body condition.

6.3 METHODS

6.3.1 Field examinations

Morphometric data was collected under anaesthesia from 137 adult wild platypuses (74 males, 63 females) captured in the Inglis Catchment between 29/8/11 and 28/8/13

(Chapter 2). Ultrasound images of tail fat were collected from 100 of these platypuses (54 males, 46 females).

Morphometrics consisted of:

- Tail Volume Index (Grant and Carrick, 1978) (1 = very good, 5 = very poor)..
- Tail length (mm) - distance from the tip of the tail (not including length of hair cover) to the caudal muscles of the body.
- Tail depth at midpoint along length measured with vernier callipers (mm).
- Tail width at midpoint along length measured with vernier callipers (mm).
- Body mass (kg) measured with Rapala® digital scales.
- Total Body length (cm) using a tape measure (tip of bill to tip of tail, measured over dorsum).
- Bill length without shield (mm) was assessed using vernier calipers.
- Bill width (mm) at its widest point was assessed using vernier calipers.

Figure 6.1. Platypus (*Ornithorhynchus anatinus*) measurements and assessments in the Inglis Catchment, Tasmania, a) body mass after subtraction of holding sack mass, b) TVI, c) bill width, d) bill length, e) total body length, f) and g) tail length. **Photos: Helen Robertson, Christina Shaw and Geoff Dutton.**

The following ultrasonographic images were collected using an Aloka Prosound 2® portable ultrasound unit and a 4cm linear transducer at 10MHz placed on the ventral aspect of the tail and the platypus in dorsal recumbency:

- Cross-section of the right half of the tail just caudal to the cloaca (referred to as tail base).

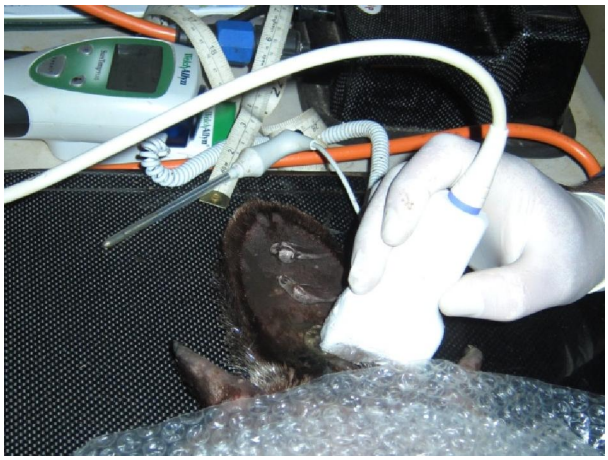
- Cross-section of the right half of the tail at the mid-point along its length (referred to as mid-tail).
- Cross-section of the right half of the tail at a point ¾ of its length from the base to the tip (referred to as ¾-tail).

The following ultrasonographic images were collected using a Signostics Speqview® hand-held ultrasound unit by sweeping the probe across the ventral aspect of the tail with the unit in M-mode:

- Cross section of the tail at the mid-tail.

Data was only analysed from the first capture of each platypus except where mentioned in relation to the repeatability of measurements and comparison of data from the two ultrasound units, where recapture data was also used.

Figure 6.2 Ultrasonography of tail base with the anaesthetised platypus in dorsal recumbency and ultrasound gel in place at mid-tail and ¾-tail. **Photo: Christina Shaw**



6.3.2 Data collected from ultrasound images

Foxit Phantom® pdf software area and distance measure tools were used on Aloka Prosound 2® ultrasound images to determine values for the following parameters: cross

sectional fat area at the tail base, cross sectional fat area at the mid-tail, cross sectional fat area at the ¾-tail level, fat depth adjacent to the spinal column at the mid-tail (mid-tail fat depth). The Foxit Phantom® pdf software distance measure tool was used on Signostics Speqview® images to determine mid-tail fat depth values.

6.3.3 Repeatability of body measures

The 12 recaptures consisted of eight platypuses that were recaptured once and two platypuses that were recaptured twice. The correlation coefficient between the first two sets of records for these ten platypuses was calculated using Microsoft Excel® for total body length, tail length, bill width and bill length. In addition, the correlation coefficient of mid-tail fat depth measurements using the Aloka Prosound 2® and Signostics Speqview® at the same examination was calculated for the 11 individuals on which both techniques were used.

6.3.4 Necropsy of road kill platypuses

Separate to the live capture and release field study, two platypuses were reported as roadkill by members of the public were examined. The tails of these platypuses were examined as part of necropsies for cause of death and general health assessment. No data was collected from these individuals, but anatomical observations were recorded.

6.3.5 Development of body condition indices based on body mass and body size

A body condition index was developed using methods similar to those of Peig and Green (2009), based on the assumption that for adult platypuses of the same body

condition there is a proportional relationship between body mass and certain linear measures of body size (LM), and the following mathematical rearrangements:

Body mass = $k*LM^n$

→ ln (body mass) = ln k +n(ln LM)

→ n = gradient of regression line for ln LM (x axis) against ln (body mass) (y axis).

& $k = e^{(\text{intercept of regression line with ln (body mass) axis})}$

Relationships were investigated between body mass and the following LMs: bill length without shield (bill length), bill width, tail length and total body length for males, females, and the overall study population. Regression lines were plotted by OLS regression on LM, by OLS regression on body mass and by RMA regression to demonstrate the different regression lines obtained, using Microsoft Excel and the computer program RMA (Bohonak and van der Linde, 2004). Consistent with the methods of Peig and Green (2009) n_i was chosen to be the gradient of the RMA regression for each LM. The amount of variation (coefficient of determination; r^2) of body mass explained by each LM^{ni} in isolation was determined using Microsoft Excel (2010). The homogeneity-of-slopes model (Statistica 8.0, Statsoft Inc., Tulsa OK USA) was used to determine whether there was a significant difference between the regression lines for males and females for each ln LM versus ln body mass regression. Multiple regression (Statistica 8.0, Statsoft Inc., Tulsa OK USA) of the LM^{ni}s against body mass was used to determine the model based on the LM^{ni}s that best explained changes in body mass for males, females and the overall population. Two body condition indices were developed based on body mass and LMs.

The process used to create the two body condition indices is summarised by the following ten steps:

Step 1. Assume that for individuals of the same body condition there is a linear relationship between body mass and LM^n, i.e. body mass = $k*LM^n$.

Step 2. Assume variation from $k*LM^n$ of an observed value for body mass is due to a difference in body mass.

Step 3. Check the repeatability of LMs where possible, as an indication of measurement error or real variation. Do so for a range of LMs if they are available.

Step 4. For males, females and the overall population, determine the value of n for an individual of average body condition using available observed data by determining the gradient of the line of best fit through a plot of ln (body mass) against ln LM. Do so for a range of LMs if they are available.

Step 5. Check the r^2 values of ln (body mass) and ln LM for males, females and the overall population. Do so for a range of LMs if they are available.

Step 6. Determine whether values of n for males, females and the overall population are similar using homogeneity of slopes assessment (Statistica 8.0, Statsoft Inc., Tulsa OK USA). Do so for a range of LMs if they are available.

Step 7. Perform standard and forward stepwise multiple regression of body mass against all LM^{ni} for all LMs (i) examined.

Step 8. Choose the most appropriate LM upon which to base a body condition index based on the following criteria: n is within the usual biologically explainable range (2.5-3.2; Green, 2001), r^2 values of ln (body mass) and

ln(LM) is high, strong correlations between body mass and LM^n found on multiple regression, and LM repeatability is high. Choose separate LMs for males and females if the n values for the two sexes are not similar (see Step 6).

Step 9. Create a body condition index = (C*body mass)/ LM^n, where C is a correction factor used to create condition index values in a manageable range. Do so separately for males and females if indicated in Step 8.

Step 10. Interpret body condition index values as follows: higher value = better body condition, lower value = poorer body condition.

6.3.6 Development of body condition indices based on morphometrics and data from ultrasound images

Tail fat volume in cm^3 for each platypus was calculated, using area measurements in cm^2 and tail length in cm, as follows:

Tail fat volume = ((2* tail base fat area) + (3 * tail fat area) + (2 * ¾ tail fat area))*(tail length/8) (See Appendix G for derivation of this formula)

The relationship between fat volume and bill width, bill length and total body length was investigated using log/log RMA regression (RMA for JAVA). The relationship of tail fat volume to tail length was not investigated because the latter was used to calculate the former. Two body condition indices incorporating tail fat volume were developed.

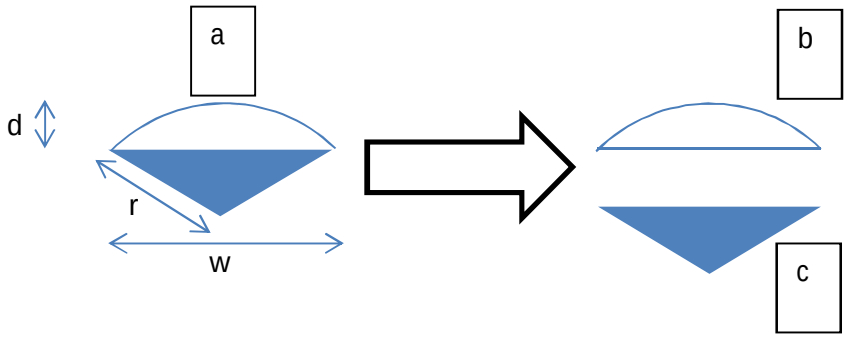
The relationships between 1) tail fat volume and mid-tail fat area, 2) mid-tail fat area and mid-tail fat depth, 3) tail fat volume and mid-tail fat depth and 4) mid-tail fat depth

and total body length were investigated. Two body condition indices incorporating mid-tail fat depth were developed.

6.3.7 Calculation of TFI

The TFI statistic developed by Temple-Smith was calculated using the sector calculator at http://www.cleavebooks.co.uk/scol/calsect.htm, making the assumption that the cross sectional area of the tail can be approximated to a segment of a circle. Given the parameters of tail width (w) and tail depth (d), the online calculator worked out the area of the circle sector that would contain this segment (Figure 6.3a). The radius of the circle which would contain this segment was calculated using the formula: $r = (w^2/8d) + (d/2)$. A triangular area (Figure 6.3c) with two sides r in length and one side w in length [(r-d)*(w/2)] was subtracted from the segment area to give the sector area (Figure 6.3b).

Figure 6.3. Areas calculated for determination of TFI



6.3.8 Comparison of body condition indices

In order to investigate whether the body condition indices were independent of body size, coefficient of determination (r^2) and probability levels (p) were determined between the body condition indices and each of total body length and bill width

(Statistica 8.0, Statsoft Inc., Tulsa OK USA). In addition, on the basis that if two or more body condition indices were accurately assessing body condition, they are likely to be correlated with each other, the coefficient of determination (r^2) and probability levels (p) were determined between all of the body condition indices (Statistica 8.0, Statsoft Inc., Tulsa OK USA). Changes in body condition indices, body mass and total body length were investigated by grouping results from January-March, April-June, July-September and October-November over the two years of fieldwork, using the methods and trimonthly groupings of Temple-Smith (1973). Mean values were calculated for each parameter in each time period and the distribution of values were compared between time periods using a Mann Whitney U test (Statistica 8.0, Statsoft Inc., Tulsa OK USA).

6.4 RESULTS

6.4.1 Ultrasound examinations

Examples of the ultrasound images collected using the Aloka Prosound 2® portable ultrasound unit and the Signostics Speqview® hand-held ultrasound unit are shown in Figures 6.4 and 6.5, respectively.

Figure 6.4. Mid-tail cross-sectional ultrasound images using Aloka Prosound 2® a) adult female - TVI 5, b) adult female - TVI 4, c) adult female TVI - 2, d) adult male - TVI 5, e) adult male - TVI 4, f) adult male TVI 2, g) adult male – TVI 2, h) adult male TVI 1, i) image e with fat area and mid-tail fat depth measuring tools.

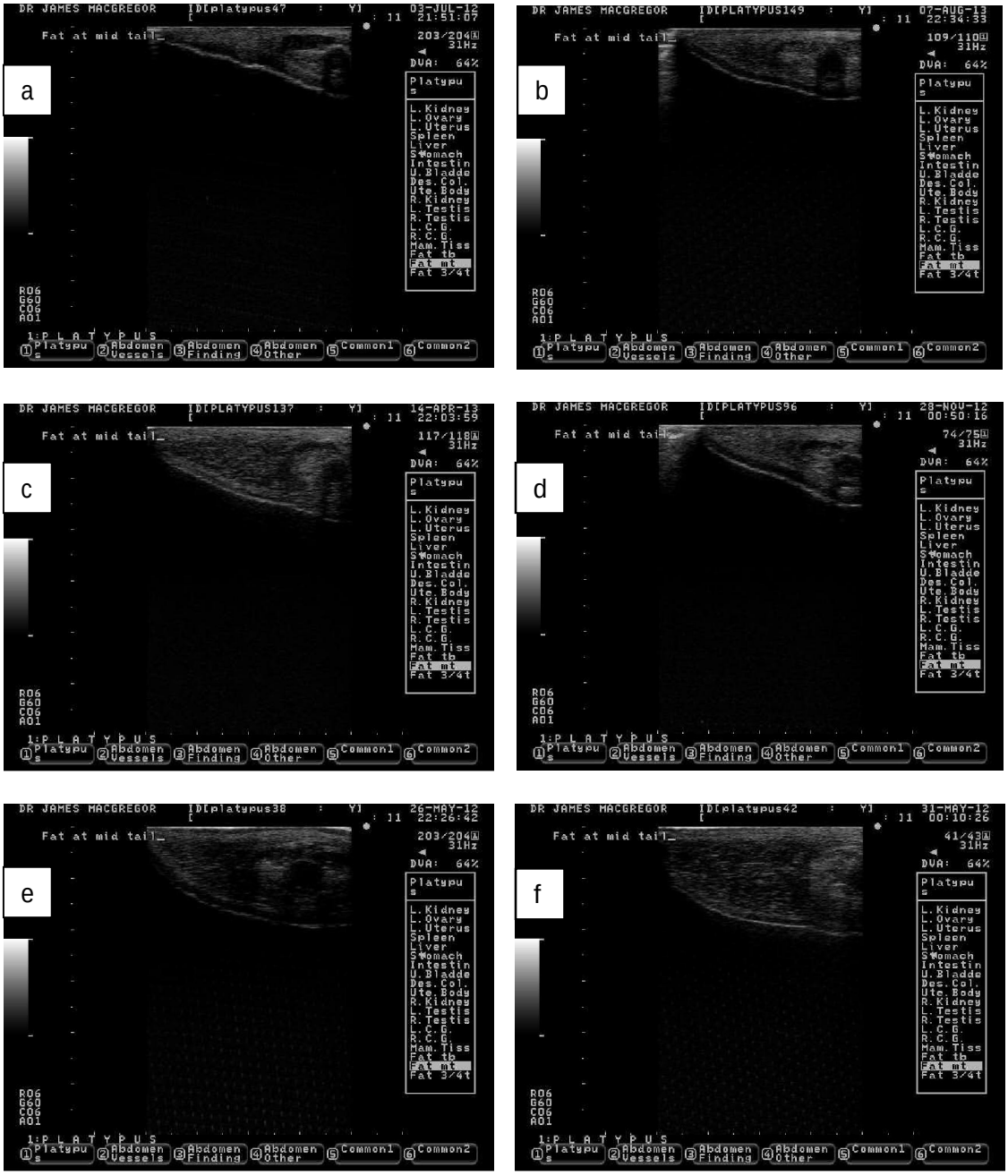


Figure 6.4 (Cont'd). Mid-tail cross-sectional ultrasound images using Aloka Prosound 2® a) adult female - TVI 5, b) adult female - TVI 4, c) adult female TVI - 2, d) adult male - TVI 5, e) adult male - TVI 4, f) adult male TVI 2, g) adult male – TVI 2, h) adult male TVI 1, i) image e with fat area and mid-tail fat depth measuring tools.

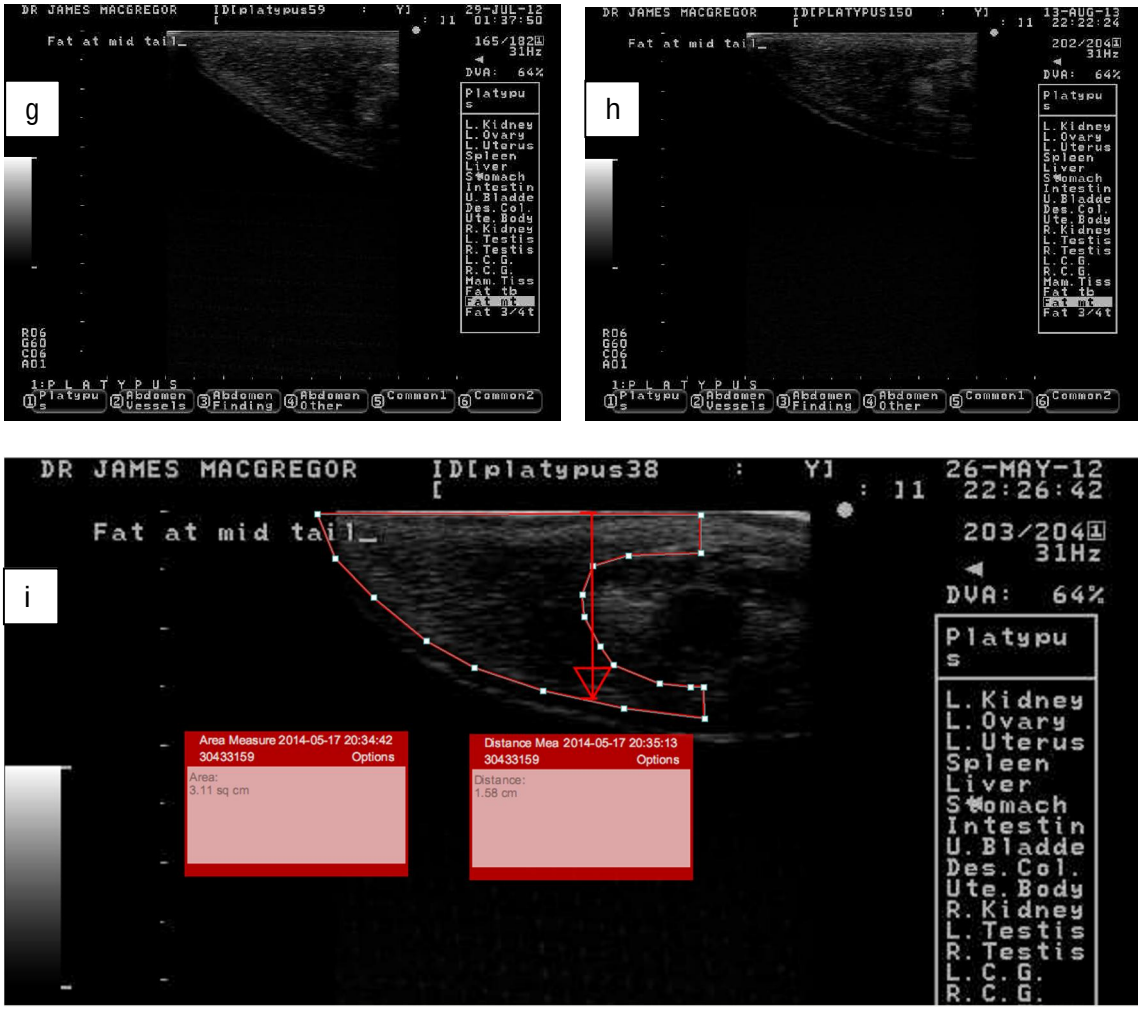
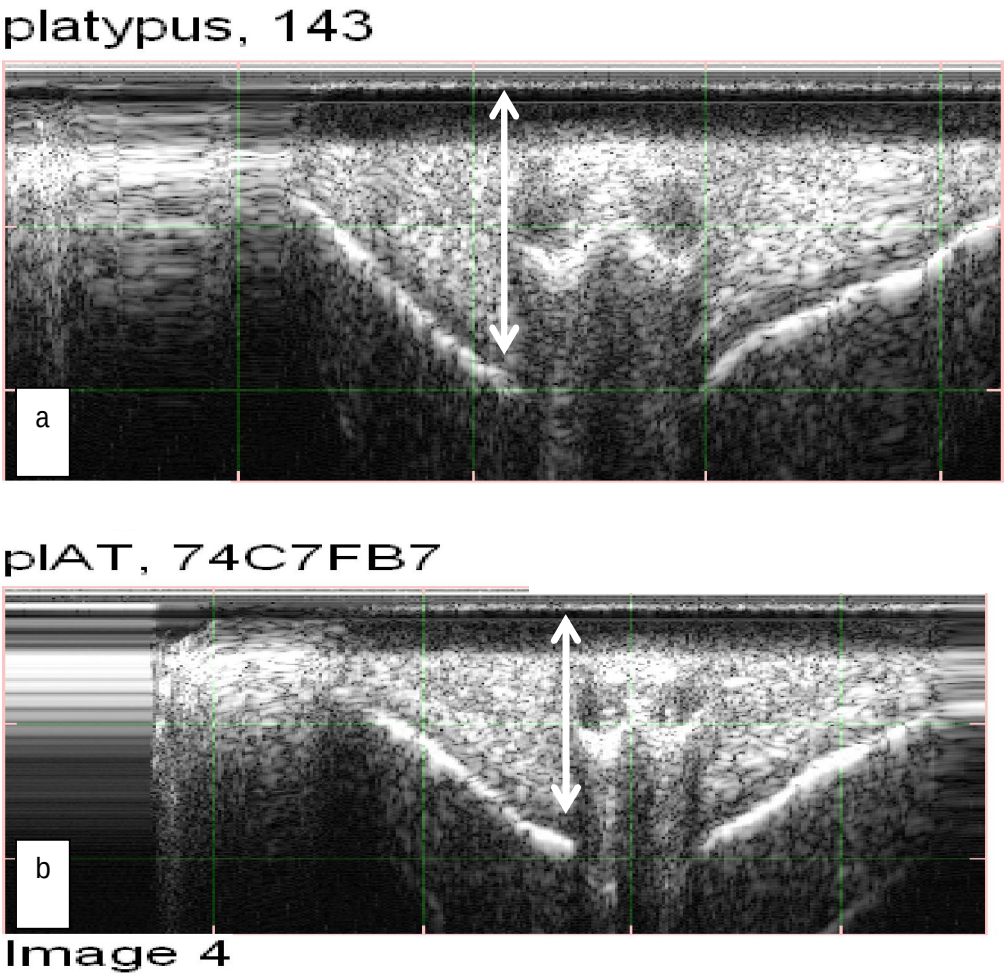


Figure 6.5. Mid-tail fat depth images using Signostics Speqview® a) adult female - TVI 4, b) juvenile male – TVI 5.



6.4.2 Repeatability of body measures

For all LMs, there were significant correlations between the first two recorded values fromm the ten platypuses that were captured more than once (Table 6.1). The highest r^2 values were obtained for bill width and total body length (Table 6.1; Step 3 in Section 6.3.5).

Table 6.1. Correlation coefficients for LM records between two captures in each of ten platypuses.

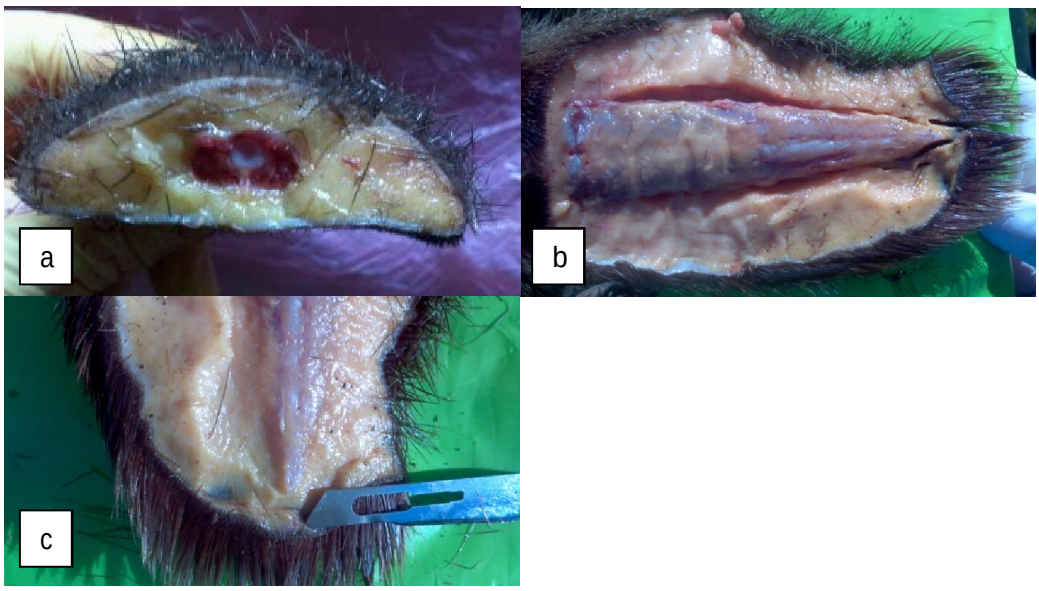
	Bill width	Bill length without shield	Total body length	Tail length
r^2	0.99	0.90	0.97	0.87
p	<0.001	<0.001	<0.001	0.001

The correlation coefficient of mid-tail fat depth measurements in the same individuals using the Aloka Prosound 2® and Signostics Speqview® was 0.97 ($p<0.001$).

6.4.3 Necropsy observations

The gross examination of the tails of two necropsied animals showed distribution of tissues similar to those seen on cross-sectional ultrasonographic examination (Figure 6.6). They also demonstrated that the spinal column has regular, smooth sides and an approximately conical shape. The tip of the tail was examined in one platypus (TVI =4). A thin layer of fat extended approximately 7mm beyond the spinal column to the tip of the tail.

Figure 6.6. Images from necropsies of two roadkill platypuses a) mid-tail cross section, b) fat dissected away from spinal column, c) tip of tail showing fat extending beyond tip of spinal column with No 22 scalpel blade for scale.



6.4.4 Development of a body condition index based on body mass and body size

For each LM for females, males and the overall study population, Figures 6.7-6.10 show ln LM plotted against ln body mass and regression lines calculated by RMA, OLS on ln body mass and OLS on ln LM (Step 4 in Section 6.3.5). The LM that gave the highest r^2 values for each of females, males and the overall population was total body length (Step 5 in Section 6.3.5). The RMA slopes of the ln/ln plots were also most similar between all three categories for total body length (Step 6 in Section 6.3.5).

Figure 6.7. Plots of ln bill width versus ln body mass of males, females and overall study population.

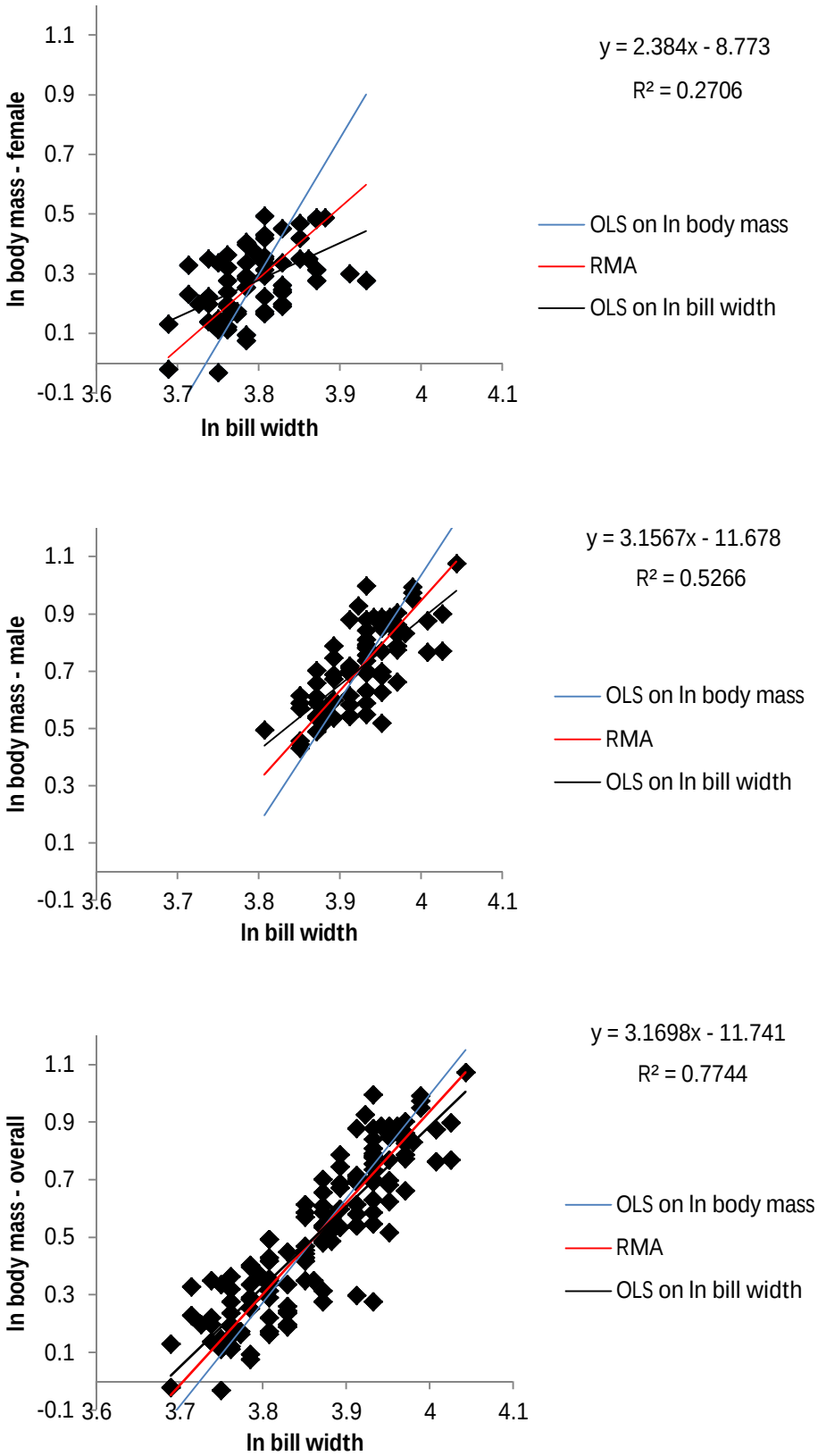


Figure 6.8. Plots of ln bill length versus ln body mass of males, females and overall study population.

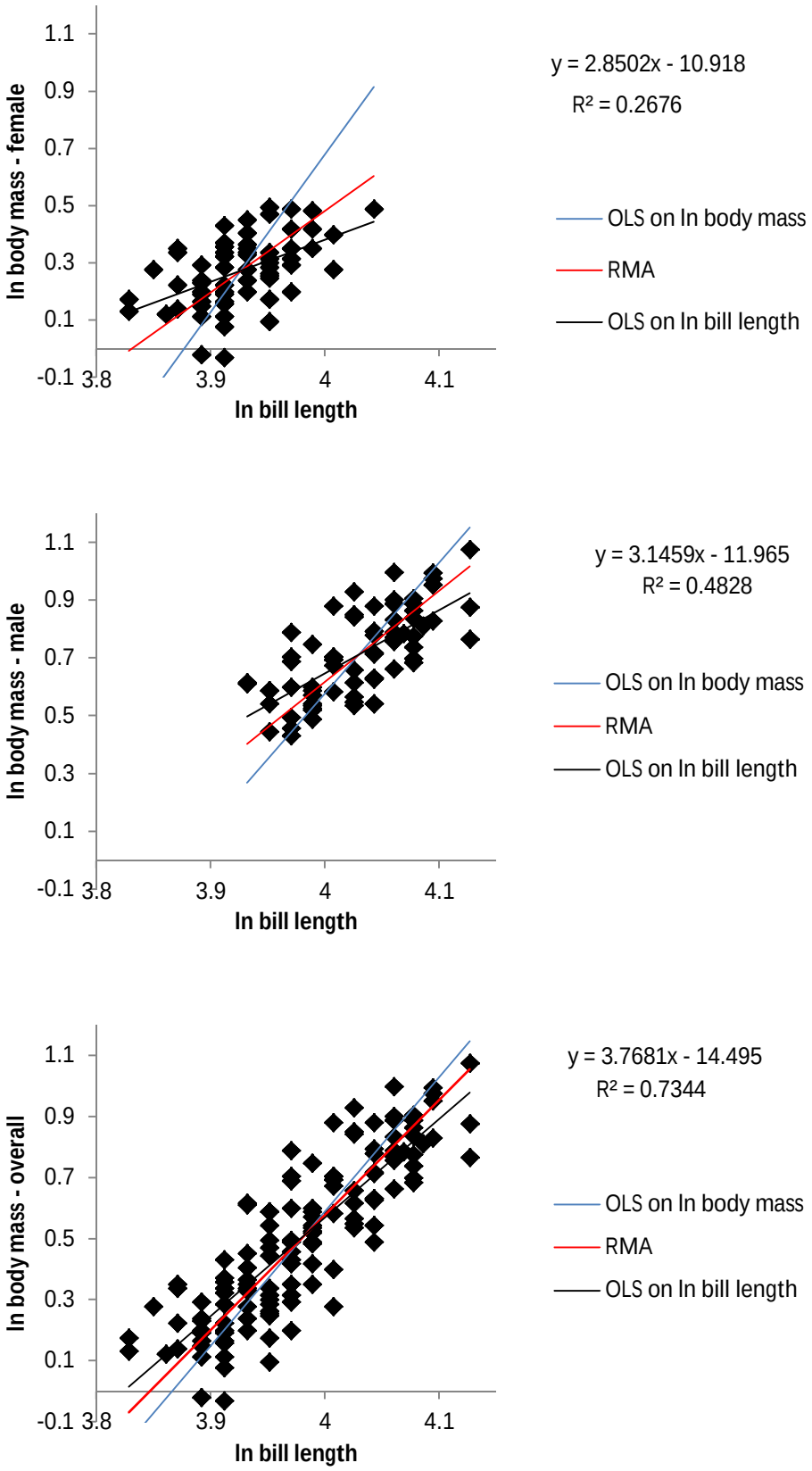


Figure 6.9. Plots of ln total body length versus ln body mass of males, females and overall study population.

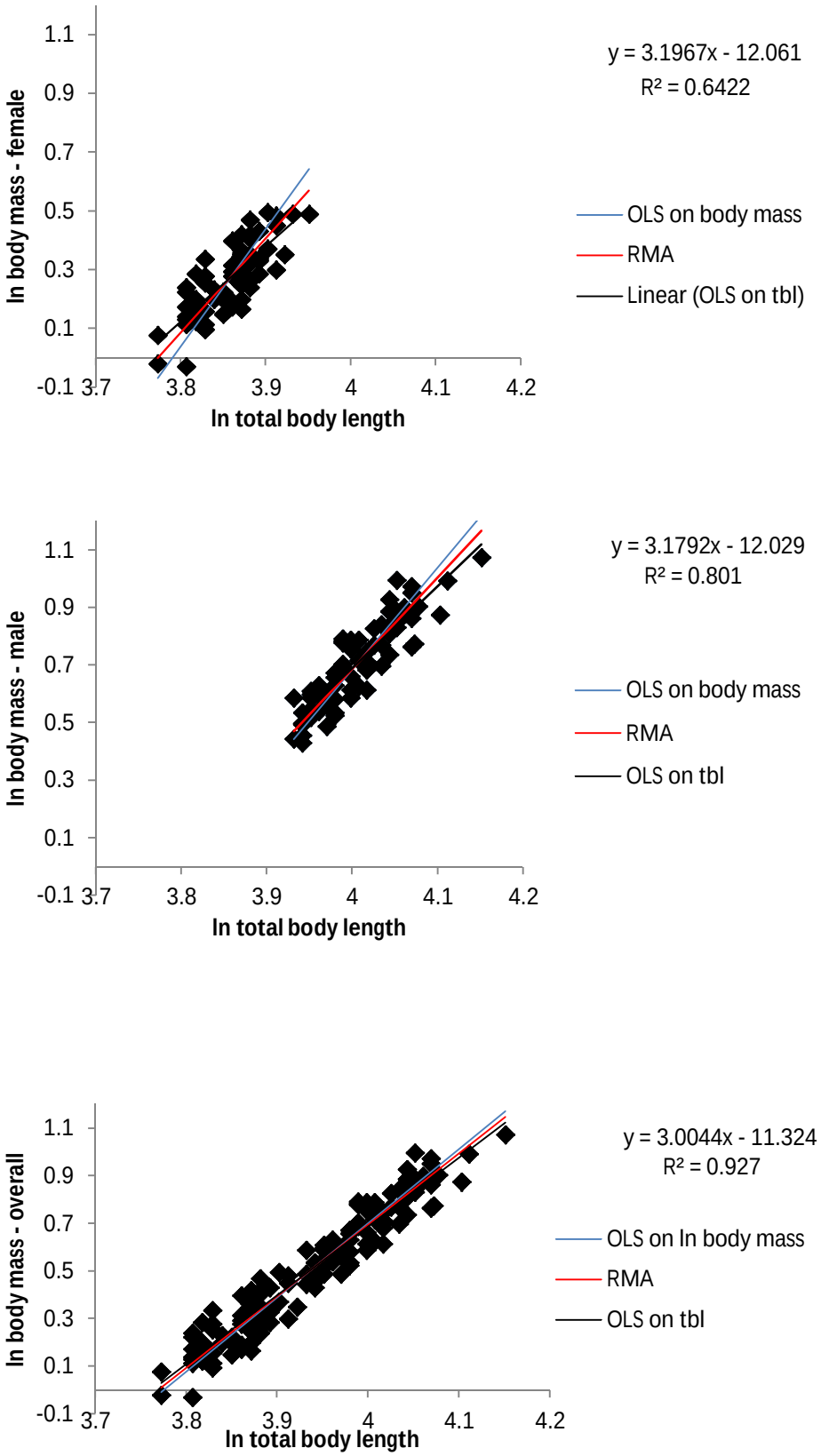


Figure 6.10. Plots of ln tail length versus ln body mass of males, females and overall study population.

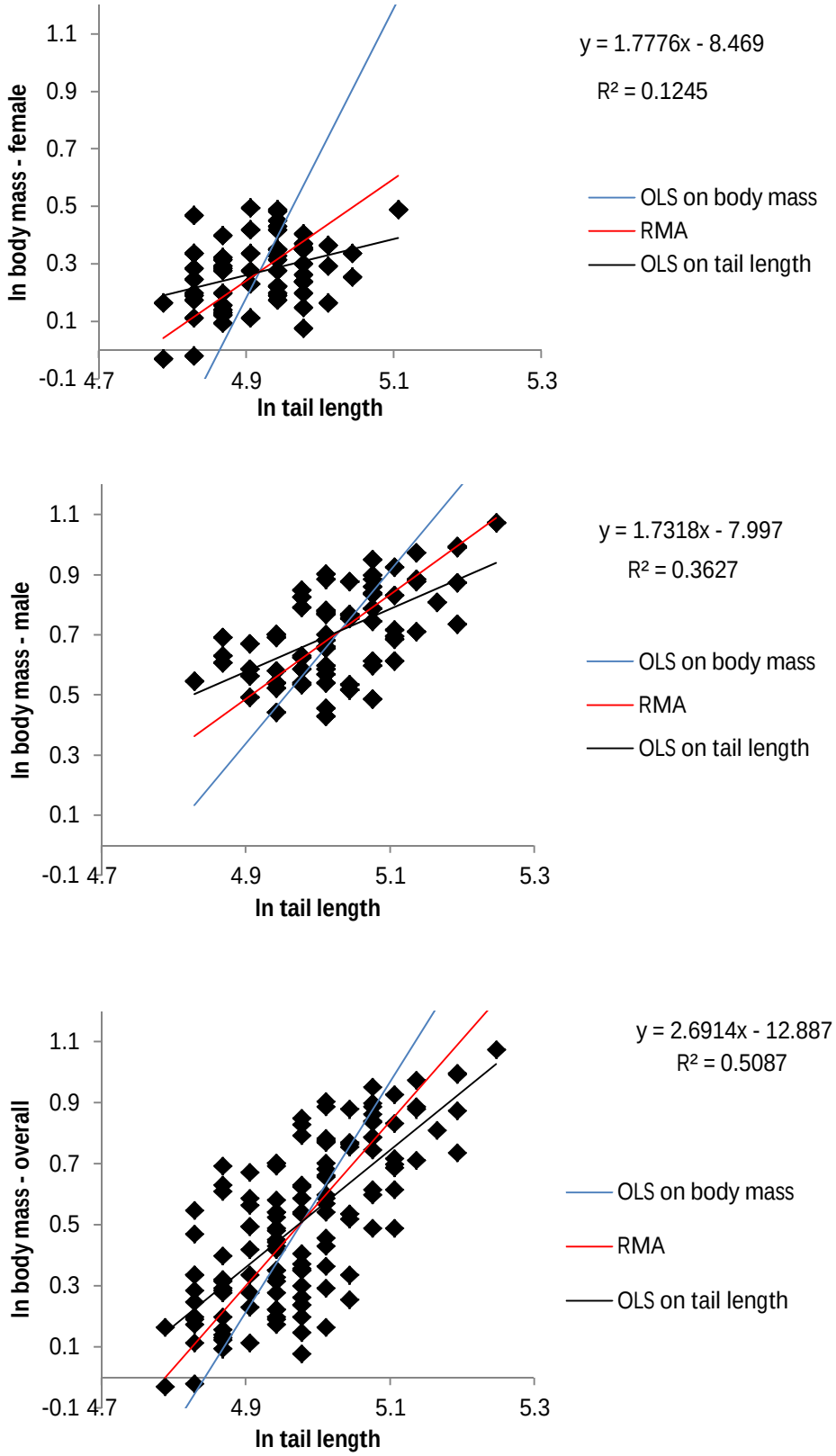


Table 6.2 shows the results of analysis of homogeneity of slopes for male and female platypuses for regression of ln body mass against ln LM (Step 6 in Section 6.3.5). There was a significant difference between the sexes in the slope of the regression line for ln body mass against bill width. The regression lines were not significantly different between the sexes for ln body mass against ln bill length, ln total body length and ln tail length. The least difference was found for ln total body length.

Table 6.2. Results of homogeneity-of-slopes model for ln body mass against ln LM and sex.

	Sum of squares	Degrees of freedom	Mean squares	F	p
ln bill width	0.081	1	0.081	7.44	0.007
ln bill length	0.028	1	0.028	2.48	0.117
ln total body length	0.004	1	0.004	0.89	0.347
ln tail length	0.009	1	0.009	1.82	0.179

Table 6.3 shows the results of the standard and forward stepwise multiple regression of body mass against each of bill width$^{3.2}$, bill length$^{3.8}$, total body length^{3} and tail length$^{2.7}$ for males, females and the overall study population (Step 7 in Section 6.3.5). The only factor that remained in each forward stepwise model, and the only factor that was significantly related to body mass, was total body length^{3}.

Table 6.3. Results of the multiple regression of ln body mass against each LM^{ni} for males, females and the overall study population.

		female	male	overall
All effects p-level	Bill width$^{3.2}$	0.955	0.246	0.196
	Bill length$^{3.8}$	0.772	0.638	0.601
	Total body length^{3}	<0.001	<0.001	<0.001
	Tail length$^{2.7}$	0.428	0.646	0.627

		female	male	overall
Forward stepwise p-level	Bill width$^{3.2}$		0.311	0.253
	Bill length$^{3.8}$			
	Total body length^{3}	<0.001	<0.001	<0.001
	Tail length$^{2.7}$			

These indications of a strong relationship between body mass and total body length^3 that appears to be at least very similar in males and females suggest that the body condition index based on the ratio of body mass to total body length^3 should be investigated in all the study animals (Step 8 in Section 6.3.5). To remove decimal places, this ratio was multiplied by 10^6, to create the body condition index "BCI 1" = 10^6 *Body mass/ total body length^3 (the factor of 10^6 doesn't affect any analysis of the results but makes the body condition values more manageable) (Step 9 in Section 6.3.5).

However, because of the presence of fat at the tip of the tail and the possibility that tail length, and hence body length, varies with body weight in the same individual, a body condition index that did not depend on total body length or tail length was developed. The logarithm plots showed that the values of r^2 for (ln bill width) and ln (bill length) plotted against ln (body mass) were similar to each other but less than that for ln (total body length) plotted against ln (body mass). There was no significant difference in the correlation slopes between males and females for bill length, but the overall slope (3.8) was outside the range of values used in most biologically explainable body condition

indices (2.5-3.2; Green, 2001). There was a significant difference in the correlation of slopes between males and females for bill width but the overall slope (3.2) was within the range of values used in most biologically explainable body condition indices (2.5-3.2; Green, 2001). The repeatability of measurement was greater for bill width (0.99) than for bill length (0.90). Multiple regression of body mass against bill width$^{3.2}$ and bill length$^{3.8}$ showed highly significant effects for both indices ($p<0.001$), but the magnitude of the effect was 24 times greater for bill width$^{3.2}$. Taking into account all of these factors, a body condition index based on the ratio of body mass to bill width$^{3.2}$, again with a conversion factor to remove decimal places, was determined for each platypus as follows: "BCI 2" = 10^6 *Body mass/ bill width$^{3.2}$.

6.4.5 Development of two body condition indices based on ultrasonographic measurements of tail fat and body size

The mean tail fat volume calculated from ultrasound image measurements for females was 60 ± 21 cm^3 and for males was 83 ± 28 cm^3. Table 6.4 shows the relationships between ln tail fat volume and ln bill width, ln bill length, and ln total body length.

Table 6.4. Results of RMA regression of ln tail fat volume against ln bill width, ln bill length, and ln total body length.

		Intercept	Slope	r^2
Bill width	female	-26.7	8.11	0.030
	male	-26.7	7.92	0.067
	overall	-15.2	5.03	0.212
Bill length	female	-30.8	8.88	0.025
	male	-27.7	7.97	0.067
	overall	-18.8	5.77	0.203
Total body length	female	-34.8	10.1	0.099
	male	-28.3	8.19	0.217
	overall	-14.1	4.64	0.295

The r^2 values were generally low, the highest being for the relationship between ln tail fat volume and ln total body length. Given the comparably high level of variance in body mass that was observed in section 6.4.4 to be explained by total body length^3, it was considered that investigation of a body condition index based on tail fat volume and a power of total body length would be appropriate. For the same reasons, and because it would make more biological sense, it was judged that use of total body length^3 as a comparative measure of body size would be most appropriate instead of 4.64 as indicated by the RMA analysis. The resultant body condition index was:

"Relative Tail Fat Volume 1" (RTFV 1) = 10^4*tail fat volume/total body length^3.

For comparison, a second body condition index based on tail fat volume was calculated as:

"Relative Tail Fat Volume 2" (RTFV 2) = 10^4*tail fat volume/billwidth$^{3.2}$.

Table 6.5. Results of RMA regression of 1) tail fat volume against mid-tail fat area, 2) mid-tail fat area against mid-tail fat depth, 3) tail fat volume against mid-tail fat depth, 4) ln mid-tail fat depth against ln total body length.

		Intercept	Slope	r^2
ln tail fat volume	female	3.129	0.9992	0.9472
vs ln mid-tail fat area	male	2.998	1.194	0.9128
	overall	3.049	1.125	0.9281
ln mid-tail fat area	female	0.6338	1.7	0.916
vs ln mid-tail fat depth	male	0.6933	1.401	0.9251
	overall	0.6529	1.542	0.9223
ln tail fat volume	female	3.762	1.698	0.8733
vs ln mid -tail fat depth	male	3.826	1.673	0.8436
	overall	3.783	1.735	0.8748
ln mid-tail fat depth	female	-22.683	5.921	0.0709
vs ln total body length	male	-19.301	4.896	0.09259
	overall	-10.288	2.674	0.1969

The relationships between 1) ln tail fat volume and ln mid-tail fat area, 2) ln mid-tail fat area and ln mid-tail fat depth, 3) ln tail fat volume and ln mid-tail fat depth shown in table 6.5 indicate that mid-tail fat depth$^{1.7}$ was well correlated with tail fat volume. ln mid-tail fat depth was poorly correlated with ln total body length. For the same reasons as earlier in this section, total body length^3 and bill width$^{3.2}$ were used as a comparative measure of body size in the body condition index "Relative Fat Depth 1" (RFD 1) = 10^6*mid-tail fat depth$^{1.7}$/total body length^3 and a second body condition index "Relative Fat Depth 2" (RFD 2) = 10^6*mid-tail fat depth$^{1.7}$/bill width$^{3.2}$ were calculated.

6.4.6 Comparison of body condition indices

Table 6.6 shows the r^2 and p values for the correlation between the body condition indices, as well as between the body condition indices and total body length and bill width. Significant correlations between either males or females and total body length

were found only for males with TVI and females with TFI - in both cases r^2 was 0.08. Significant correlations between either males or females and bill width were found for females with RTFV 2 and both males and females with BCI2 – r^2 was 0.12, 0.14 and 0.46, respectively. Significant overall correlations with total body length were found for TFI and RFD 1 – r^2 was 0.12 and 0.05, respectively. Significant overall correlations with bill width were found for TFI, RTFV 2, RFD 1, RFD 2, and BCI 2 – all with r^2 of less than 0.11.

TVI, TFI, RTFV 1, RTFV 2, RFD 1 and RFD 2 were all significantly correlated for males, females and the overall sample population. BCI 1 and BCI 2 were significantly correlated in 17/21 and 16/21 comparisons. The r^2 values were highest for correlations between the body condition indices involving ultrasound measurements of fat (RTFV 1, RTFV 2, RFD 1 and RFD 2). Figure 6.11 illustrates the following correlations:

1) TVI vs RFD 2 for males – the highest r^2 value for a comparison with TVI
2) TVI vs TFI for females
3) RFD 2 vs RTFV 2 for males – the highest r^2 value for a comparison not involving either both RTFV 1 and RTFV 2 or both RFD 1 and RFD 2.
4) RFD 1 vs total body length for males and females – a significant overall correlation
5) TVI vs total body length for males – a significant correlation

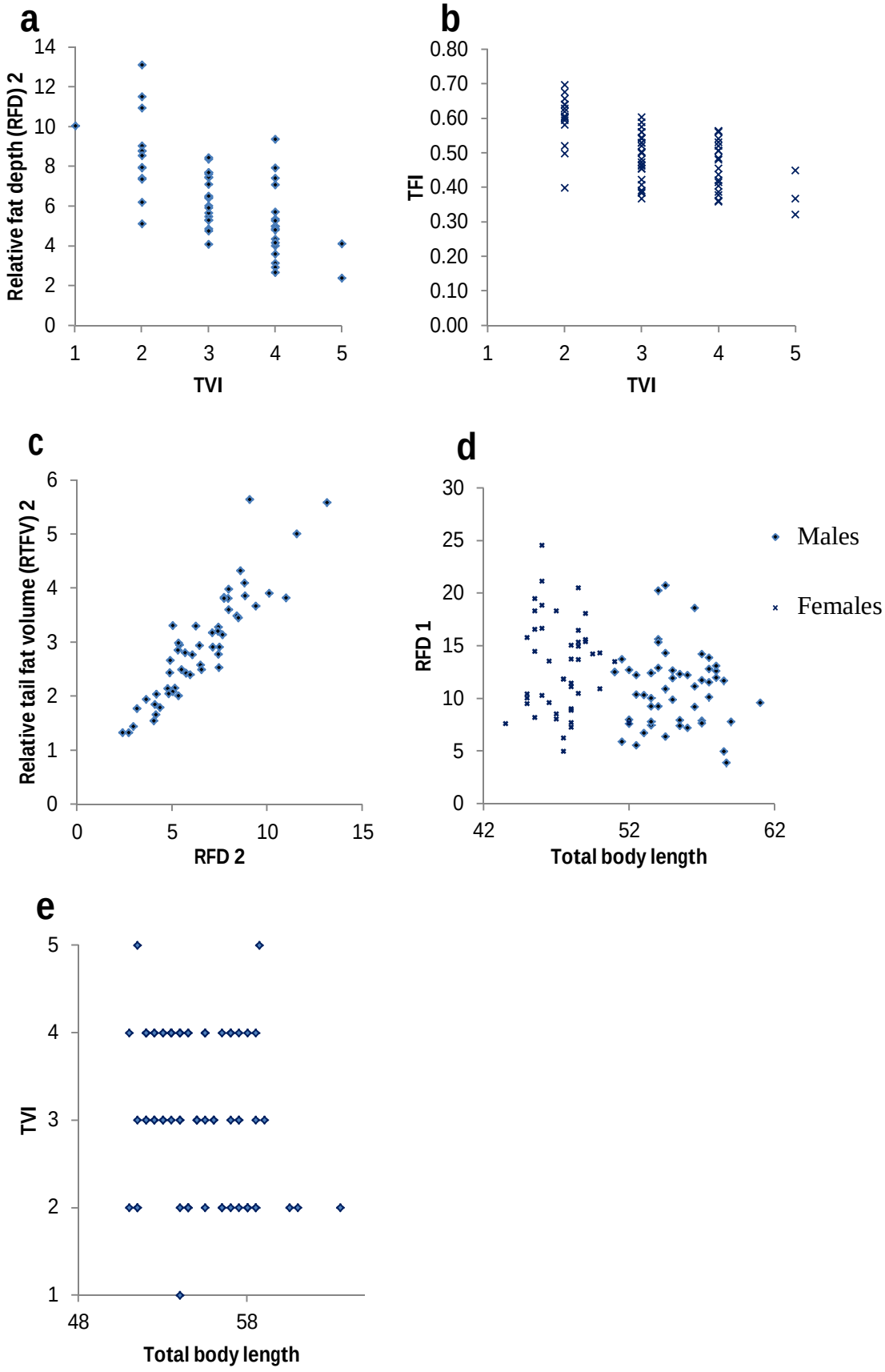
Table 6.6. Correlations between the body condition indices and between the body condition indices and total body length and bill width (r^2, p value and n).

		TVI				TFI				RTFV 1				RTFV 2			
		r²	p	n	+/-	r²	p	n	+/-	r²	p	n	+/-	r²	p	n	+/-
Total	f	0.03	0.159	63	-	0.08	**0.027**	62	+	0.00	0.961	46	-	0.00	0.697	46	+
body	m	0.08	**0.017**	74	-	0.01	0.361	72	+	0.02	0.364	54	+	0.05	0.101	54	+
length	all	0.02	0.133	137	-	0.12	**0.001**	134	+	0.02	0.146	100	-	0.001	0.69	100	-
Bill	f	0.01	0.404	63	-	0.03	0.19	62	+	0.00	0.968	46	+	0.06	0.104	46	-
width	m	0.01	0.32	74	-	0.00	0.863	72	+	0.00	0.958	54	-	0.02	0.292	54	-
	all	0.01	0.39	137	-	0.09	**0.001**	134	+	0.03	0.101	100	-	0.05	**0.033**	100	-
	f	0.36	**<0.001**	62	-												
TFI	m	0.33	**<0.001**	72	-												
	all	0.31	**<0.001**	134	-												
	f	0.24	**0.001**	46	-	0.50	**<0.001**	45	+								
RTFV	m	0.44	**<0.001**	54	-	0.54	**<0.001**	52	+								
1	all	0.31	**<0.001**	100	-	0.38	**<0.001**	97	+								
	f	0.22	**0.001**	46	-	0.49	**<0.001**	45	+	0.86	**<0.001**	46	+				
RTFV	m	0.43	**<0.001**	54	-	0.48	**<0.001**	52	+	0.95	**<0.001**	54	+				
2	all	0.31	**<0.001**	100	-	0.39	**<0.001**	97	+	0.87	**<0.001**	100	+				
	f	0.30	**<0.001**	46	-	0.52	**<0.001**	45	+	0.85	**<0.001**	46	+	0.80	**<0.001**	46	+
RFD	m	0.45	**<0.001**	54	-	0.60	**<0.001**	52	+	0.79	**<0.001**	54	+	0.66	**<0.001**	54	+
1	all	0.34	**<0.001**	100	-	0.37	**<0.001**	97	+	0.83	**<0.001**	100	+	0.73	**<0.001**	100	+
	f	0.26	**<0.001**	46	-	0.47	**<0.001**	45	+	0.68	**<0.001**	46	+	0.88	**<0.001**	46	+
RFD	m	0.46	**<0.001**	54	-	0.56	**<0.001**	52	+	0.77	**<0.001**	54	+	0.91	**<0.001**	54	+
2	all	0.33	**<0.001**	100	-	0.37	**<0.001**	97	+	0.72	**<0.001**	100	+	0.85	**<0.001**	100	+
	f	0.02	0.245	63	-	0.10	**0.011**	62	+	0.17	**0.004**	46	+	0.07	0.078	46	+
BCI1	m	0.06	**0.034**	74	-	0.27	**<0.001**	72	+	0.34	**<0.001**	54	+	0.22	**<0.001**	54	+
	all	0.04	**0.020**	137	-	0.14	**<0.001**	134	+	0.25	**<0.001**	100	+	0.14	**<0.001**	100	+
	f	0.01	0.396	63	-	0.04	0.099	62	+	0.05	0.152	46	+	0.25	**<0.001**	46	+
BCI2	m	0.12	**0.003**	74	-	0.18	**<0.001**	72	+	0.23	**<0.001**	54	+	0.46	**<0.001**	54	+
	all	0.05	**0.008**	137	-	0.1	**<0.001**	134	+	0.09	0.002	100	+	0.32	**<0.001**	100	+

Table 6.6 (cont'd). Correlations between the body condition indices and between the body conditio indices and total body length and bill width (r^2, p value and n).

		RFD 1				RFD 2				BCI1				BCI2			
		r^2	p	n	+/-	r^2	p	n	+/-	r^2	p	n	+/-	r^2	p	n	+/-
Total body length	f	0.02	0.647	46	-	0.00	0.999	46	-	0.05	0.075	63	-	0	0.98	63	-
	m	0.00	0.715	54	+	0.00	0.703	54	+	0.01	0.360	74	-	0.03	0.132	74	+
	all	0.07	**0.010**	100	-	0.03	0.115	100	-	0.02	0.123	137	-	0.01	0.211	137	+
Bill width	f	0.00	0.412	46	-	0.12	**0.018**	46	-	0.01	0.506	63	-	0.46	**<0.001**	63	-
	m	0.01	0.427	54	-	0.06	0.073	54	-	0.00	0.809	74	+	0.14	**<0.001**	74	-
	all	0.08	**0.005**	100	-	0.11	**<0.001**	100	-	0.00	0.438	137	-	0.07	**0.002**	137	-
TFI	f																
	m																
	all																
RTFV 1	f																
	m																
	all																
RTFV 2	f																
	m																
	all																
RFD 1	f																
	m																
	all																
RFD 2	f	0.87	**<0.001**	46	+												
	m	0.90	**<0.001**	54	+												
	all	0.87	**<0.001**	100	+												
BCI1	f	0.14	**0.011**	46	+	0.04	0.158	46	+								
	m	0.37	**<0.001**	54	+	0.26	**<0.001**	54	+								
	all	0.24	**<0.001**	100	+	0.13	**<0.001**	100	+								
BCI2	f	0.08	0.052	46	+	0.3	**<0.001**	46	+	0.18	0.001	63	+				
	m	0.17	**0.002**	54	+	0.39	**<0.001**	54	+	0.18	**<0.001**	74	+				
	all	0.09	**0.002**	100	+	0.3	**<0.001**	100	+	0.17	**<0.001**	137	+				

Figure 6.11. Graphic illustrations of selected correlations from Table 6.6.



Trimonthly changes in total body length, body mass and body condition indices are illustrated in Figures 6.12 and 6.13 for males and females, respectively. All of the male body condition indices except BCI 2 indicated maximum mean body condition occurred in the July-September period. All of the female body condition indices except TVI indicated maximum mean body condition occurred in the January-March period. Minimum and maximum trimonthly values were significantly different for all parameters except TVI in females and for TVI, TFI, RFD 1, RTFV 1 and RFD 2 in males (Table 6.7). Mean male TVI was lower in July-September than October-November (Table 6.8). All other body condition indices in males except BCI 2 showed a decrease in mean value from July-September to October-November. The distribution of results in these two time periods was significantly different for all indices except BCI 1 and BCI 2. Mean female TVI for females was lower in January-March than April-June but the distribution of results was not significantly different. All other body condition indices showed a decrease in mean value from January-March to April-June and the distribution of results was significantly different in these two time periods for all of these except BCI 2. Mean female total body mass and total body length were significantly higher in October-November than April-June.

Figure 6.12. Trimonthly mean body condition index, body mass and total body length values for males.

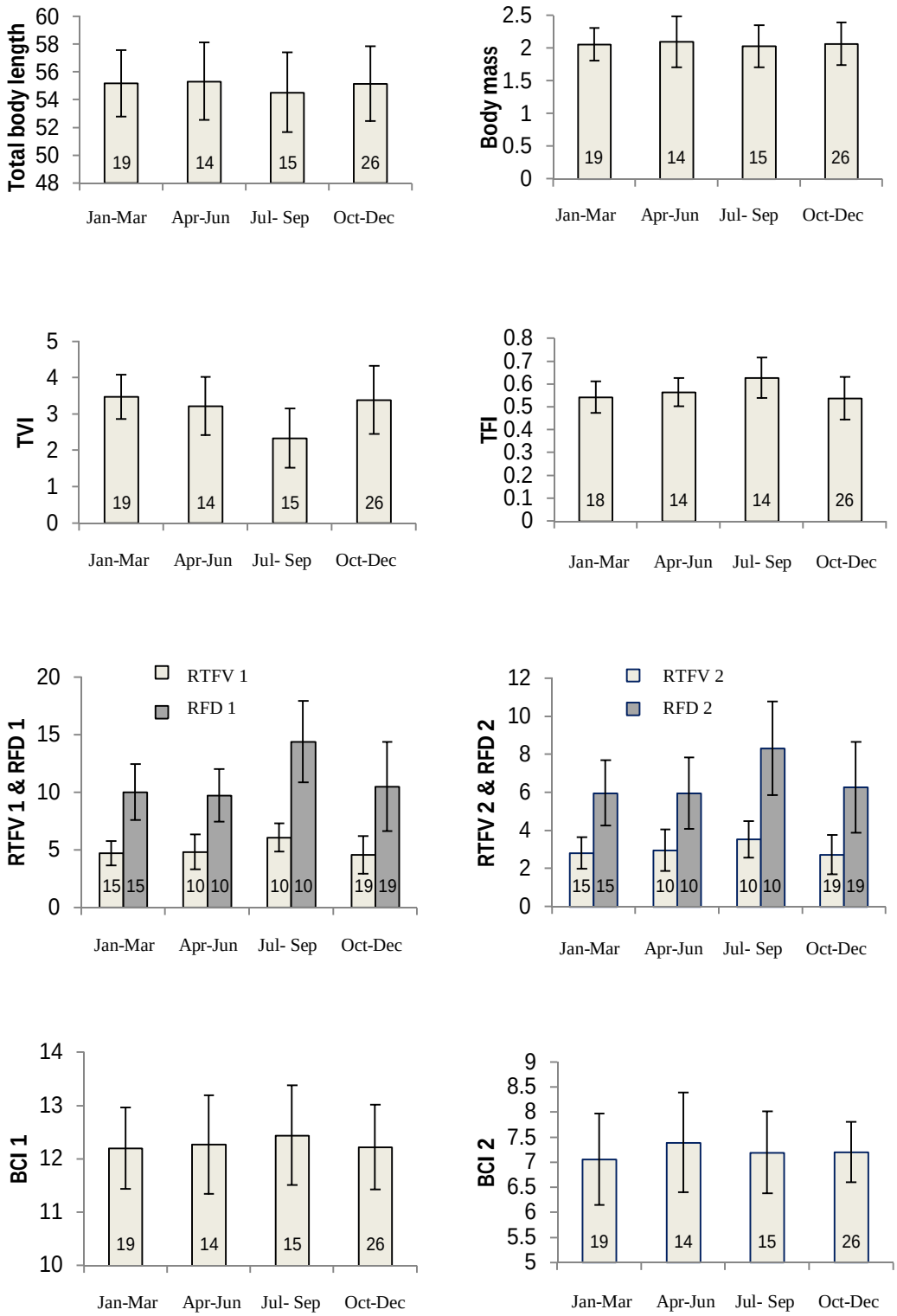


Figure 6.13. Trimonthly mean body condition index, body mass and total body length values for females. Sample size shown in columns.

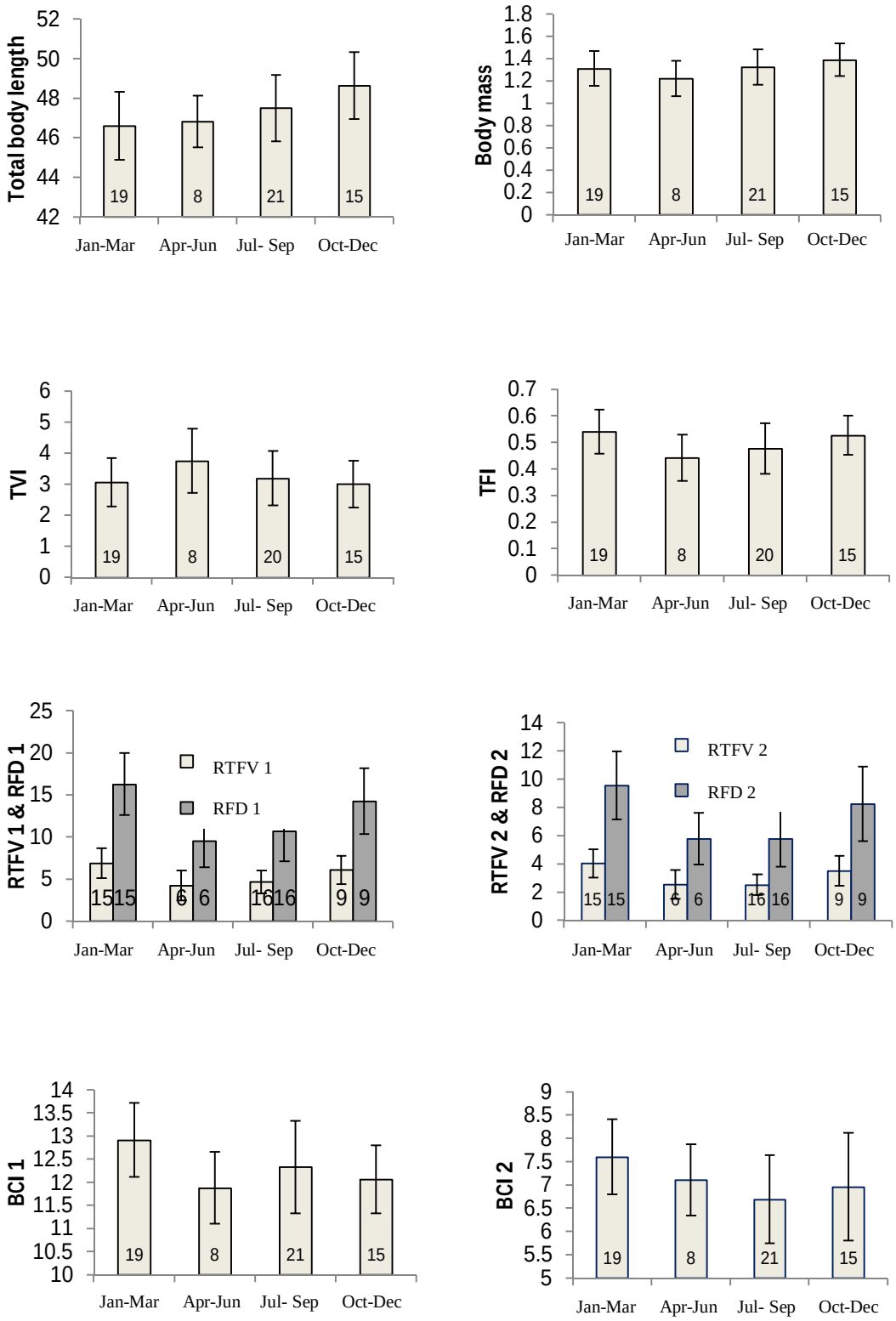


Table 6.7. Results of Mann Whitney U tests on the maximum and minimum trimonthly periods for each parameter for each sex.

	Males			Females		
	Period of min mean value	Period of max mean value	p	Period of min mean value	Period of max mean value	p
Total body length	JAS	AMJ	0.425	JFM	OND	**0.006**
Body mass	JAS	AMJ	0.715	AMJ	OND	**0.040**
TVI	JAS	JFM	**<0.001**	OND	AMJ	0.101
TFI	OND	JAS	**0.009**	AMJ	JFM	**0.016**
BCI1	JFM	JAS	0.471	AMJ	JFM	**0.008**
RTFV 1	OND	JAS	**0.019**	AMJ	JFM	**0.011**
RFD 1	AMJ	JAS	**0.002**	AMJ	JFM	**0.002**
BCI 2	JFM	AMJ	0.255	JAS	JFM	**0.002**
RTFV 2	JFM	JAS	0.062	OND	JFM	**0.001**
RFD 2	JFM	JAS	**0.019**	AMJ	JFM	**0.005**

Table 6.8. Results of Mann Whitney U tests for body size and condition parameters between July-September and October-November in males and January-March than April-June in females.

	Males			Females		
Body condtion	Time period 1	Time period 2	p	Time period 1	Time period 2	p
Total body length	OND	JAS	0.565	AMJ	JFM	0.815
Body mass	OND	JAS	0.738	AMJ	JFM	0.238
TVI	OND	JAS	**0.002**	AMJ	JFM	0.106
TFI	OND	JAS	**0.009**	AMJ	JFM	**0.016**
BCI1	OND	JAS	0.398	AMJ	JFM	**0.008**
RTFV 1	OND	JAS	**0.019**	AMJ	JFM	**0.011**
RFD 1	OND	JAS	**0.019**	AMJ	JFM	**0.002**
BCI 2	OND	JAS	0.989	AMJ	JFM	0.180
RTFV 2	OND	JAS	**0.045**	AMJ	JFM	**0.014**
RFD 2	OND	JAS	**0.050**	AMJ	JFM	**0.005**

6.5 DISCUSSION

This study provides valuable insights into the performance of a range of new and existing indices of platypus body condition. This discussion will first present general comments about the results and then move on to discuss each body condition index individually.

Firstly, it should be noted that there were significant correlations between many of the body condition indices in males, female and the overall study population, as shown in Table 6.6. In fact for correlations that didn't involve BCI 1 and BCI 2, all the results were statistically significant, and 30 of the 39 correlations involving BCI 1 and BCI 2 were statistically significant. In addition, the trimonthly averages in Figures 6.12 and 6.13 show similar trends. These observations indicate that the results for the body condition indices investigated are, at least in part, measuring/assessing the same factor. The r^2 values for comparisons between RTFV 1, RTFV 2, RFD 1 and RFD 2 were generally high. This may not be surprising given that they were based on measurements from the same ultrasound images. However, r^2 values for other comparisons between body condition indices varied between 0.04 and 0.60. Figure 6.11 demonstrates the considerable amount of overlap between TVI categories when plotted against RFD 2 - the body index with which it is most correlated-and TFI. These observations indicate that use of the body condition indices investigated, including TVI and TFI, may be able to reliably detect differences in mean values for groups of platypuses, but that use for assessing individual body condition may not be useful or may even be misleading.

Factors leading to poorer body condition are those that lead to negative energy balance, either by reduced energy intake or increased energy expenditure. Factors that may vary

seasonally and affect energy intake would include reduced prey availability due to environmental conditions and reduced foraging time during egg incubation in females. Factors that may vary seasonally and affect energy expenditure would include maintaining body temperature during winter, increased activity during the breeding season, and lactation in females (Bethge, 2001). The trimonthly mean body condition index values in this study were consistent in suggesting a general decrease in body condition from July-September to October-December in males and from January-March to April-June in females. The difference between the sexes in the timing of this likely decrease in body condition suggests that environmental factors (to which both sexes would be equally exposed) are in general not as important as reproductive factors. Because these seasonal changes have been inferred from trimonthly body condition index averages the timing of the likely overall decreases in body condition cannot be determined precisely. However, the timing of the observed decrease in trimonthly averages in males is consistent with the timing of the main breeding season in November and December, as determined in Chapter 5. Also, the timing of the observed decrease in trimonthly averages in females is consistent with the latter stages of the period of lactation in females, inferred from the timing of the breeding season.

The results of this study are similar to those of Handasyde *et al.* (2003) who observed overall decreases in body condition of both sexes during the breeding season in 28 adult males and 44 adult females in Victoria, and further decreases in females during lactation, but this study did not perform statistical analysis. Temple-Smith (1973), however, reported changes in two tail fat indices that were similar between the sexes. These indices were observed to be highest in summer, showing a small decline during autumn and early winter and an appreciable reduction in August and September.

Lactating females were observed to have lower body condition index results than non-lactating females in the same month (Temple-Smith, 1973). Temple-Smith (1973) also assessed the gradient and position of regression lines of log/log plots of body length and body mass. This is a similar approach to that used in BCI 1 and BCI 2 in this investigation. Temple-Smith (1973) found the changes in the regression lines between July-October and January-April to be consistent between males and females, with the gradients being the same but the position being significantly different. These results suggested that body mass in both sexes tended to be lower in July-October than January-April. Bimonthly and trimonthly body mass means at different field sites were also highest during summer and lowest during spring. However, it was also noted that lactating females showed "an appreciable loss of body condition in the latter months of lactation (January to April) when compared to non-lactating, anoestrous females caught during the same period, and were consequently not included in female data for analysis" (Temple-Smith, 1973, P.49). In a smaller study of the introduced wild population on Kangaroo Island by Serena and Williams (1997), it was observed that the mean body weight and body condition of adult male and adult female platypuses was higher in December than in October-November. Grant and Carrick (1978) observed trends towards higher body mass and lower TVI in summer.

Hulbert and Grant (1983) estimated total body fat by measurement of tritiated water space. They also used water displacement to measure tail volume and expressed this as a percentage of body volume for each platypus (relative tail volume). In adult platypuses of both sexes (n=9 males, n=30 females), relative tail volume was lower in September than in February, but there was only a minimal change in total body water (Hulbert and Grant, 1983). This may be a result of the tritiated water technique, or may

indicate that the distribution of fat with the platypus varies seasonally. In platypuses at Lake Lea, Tasmania, Munks *et al.* (2000) observed that there were no significant seasonal differences in mean body mass for either sex but, using the tritiated water technique, found a significant increasing in energy expenditure in winter for females, and a significant drop in body condition in winter for males. Also at Lake Lea, using a combination of activity logger and time-depth recorder data combined with laboratory metabolic rate data, Bethge (2001) reported that metabolic rates were higher in winter. However, there were no seasonal differences in body mass or TVI (Bethge, 2002).

An important feature of a reliable body condition index is that it is independent of the size of the animal. There were significant correlations with LMs (either total body length or bill width) in the overall study population in seven of the 16 such analyses. Four of these significant correlations occurred without any significant correlation in either of the sexes. Given the sexual dimorphism in size, this is likely to be an indication that body condition of the sexes should be analysed independent from each other and not compared – i.e. a male and a female with the same body condition index value may not have the same body condition. Correlation between the body condition indices and LMs in either males or females was significant in five of 32 analyses. While still a cause for concern about the reliability of the body condition indices involved, the r^2 values for four of these significant correlations was low. However, BCI 2 was significantly correlated with bill width for males and females and overall population, and the r^2=0.46 for females.

Differences were apparent between the results for BCI 1 and BCI 2 and those for the other body condition indices investigated in this study. One difference was the lack of

significant seasonal changes in males when analysed on a trimonthly basis (Tables 6.7 and 6.8). There are two possible reasons for this that might affect both BCI 1 and BCI 2. Firstly, these indices do not rely on an assessment of tail fat quantity which may lead to a better assessment of overall body fat. Secondly, potential changes in body fat relative to body size will be small relative to the changes in tail fat. As a result, a larger sample size may be required to demonstrate significant changes.

In addition to the factors affecting BCI 1 and BCI 2 results listed above, there are potential problems with all of the body condition indices that involve either total body length or bill width. The reasons that these LMs were selected from the four available have been explained. However, the fact that a necropsy showed 7 mm of fat at the tip of the tail opens the possibility that total body length may increase with body condition. If it is supposed that this 7 mm of fat may increase or decrease by 5 mm, this would equate to a change of approximately 1% of total body length and a change of approximately 3% of total body length^3. Hulbert and Grant (1983) found that total body fat made up on average 8.3 ± 1% (range 3.4-12.2%). Hence a change in total body length of 5mm, if it were to occur either with or without a change in body condition, would have a considerable effect on a body condition index based on total body fat/ total body length^3. In fact, if tail length does increase with body condition, this could explain a small part of the correlation between body length and body mass. Such a change in total body length and tail length is likely to have much less of an effect on assessments of tail fat because there is much more variation in tail fat measurements than in body weight measurements.

In addition to this potential variation in actual total body length, there is potential for error in measurement of total body length. In this study total body length was measured under anaesthesia using a tape measure placed above the platypus. This approach was taken as it could also be easily used in non-anaesthetised platypuses. However, the curve of the dorsal surface of the platypus complicates this measurement, as does the presence of fur at the tip of the tail (Figure 6.6). Also, it is difficult to determine whether the spine of the platypus is curved either laterally or dorsoventrally and if so, whether this curvature varies between individuals due to their placement on the examination table. If such curvature is present it would reduce the total body length measurement. This problem is likely to be greater in a non-anaesthetised platypus which could actively hunch up or bend its body as a protective behaviour, and also be complicated by a moving animal. Despite these measurement issues, the repeatability of total body length measurements in this study was 0.97. Bill width was selected as a second LM to use in novel body condition indices. While the repeatability of measurement was good for bill length and there is little chance it would change with body condition, the poor correlation of ln bill width with ln body mass in females is a concern and may explain the relatively high correlation between BCI 2 and bill width.

Subjectivity and appropriateness of approximations in the body condition indices may also be a cause of inaccuracy or bias in some of the body condition indices. These will be discussed below in the detailed sections on each body condition index.

Tail Volume Index (TVI)

TVI is currently the body condition index used almost universally by platypus researchers. It has the advantage of being a very quick method of assessing the amount of fat in the tail. But it has the disadvantage of being reasonably subjective. One obvious counter argument to suggestions in the comments below of limitations of TVI is that the limitations are actually in my ability to apply a reliable technique. However, in my experience there is often doubt about which of two adjacent scores to give a platypus. This is partly because of the lack of clear cut-off points between adjacent scores, and partly because some platypus tails do not possess all of the characteristics of a particular category. As a result, there is probably a lack of consistency within my own observations. It is also likely that different researchers will assign scores slightly differently to each other and cause bias in comparisons between studies. The effects of this are magnified by the fact that although there are five categories in the TVI scale, nearly all adult platypuses are scored as 2, 3 or 4 (Figure 6.11; Grant and Carrick, 1978; Sarah Munks, personal communication; Joanne Connolly, personal communication).

Assessment of TVI does not involve the use of body mass or LMs, and so avoids potential limitations of doing so. However, despite this there was still a significant negative correlation with body length in males. Although r^2 for this correlation was low, this correlation may suggest that there are difficulties applying the technique to individuals of different size. Also, as with all the indices that aim to measure tail fat, there is the issue that the relationship of tail fat to body condition has not been clearly demonstrated.

Tail Fat Index (TFI)

TFI was developed as part of the first reported investigation into platypus body condition (Temple-Smith, 1973). Within a few years the use of TVI was reported (Grant and Carrick, 1978) and TFI has rarely been used since. Temple-Smith (1973) reported significant positive correlations between TFI and relative tail volume in both male ($p<0.001$, $r^2=0.891$) and female ($p<0.001$, $r^2=0.829$) platypuses. The values for TFI in this study were not as widely distributed as those reported by Temple-Smith (1973), all being in the lower half of his ranges for males and females. The reason for this is not explained.

While TFI avoids the issue of subjectivity it (like the other indices relating to tail fat) suffers from the possibility that tail fat does not reliably indicate body condition, and also has four other likely issues relating to accuracy. The first issue is that TFI measures both the fat and the other tissues within the tail (spinal bones and muscles, skin, fur). Secondly TFI relates an area measure in cm^2 (cross-sectional area) to a linear measure (tail length). This leads to the potential that bigger platypuses will receive bigger scores which could at least in part be responsible for the significant positive correlation of TFI with total body length in females and overall, and with bill width overall, observed in this study. The third issue that may affect the reliability of TFI as a body condition index relates to the measurement of tail width. In my experience, the edges of the tail are often soft and have a variable thickness of fur. I find that in applying callipers it can be difficult to determine when the device is in the correct position. The last potential problem with TFI is the assumption that the cross-sectional area at the middle of the tail approximates to an arc. The differing cross-sectional shapes in Figure 6.4 demonstrate

that this assumption is not always correct. Much of the variation in tail fat cross-sectional area occurs in the dorso-ventral direction, just lateral to the spine (producing a concave, flat or convex dorsal surface), which measurements of tail depth and tail width are likely not to adequately reflect.

Relative Tail Fat Volume 1 and Relative Tail Fat Volume 2 (RTFV 1 and RTFV 2)

The use of ultrasonography in this investigation was intended to provide an accurate and objective assessment of tail fat volume. While it is my opinion that RTFV 1 and RTFV 2 provide the most reliable assessment of tail fat, it is not possible to prove this from this investigation. The uncertain relationship between tail fat and body condition is a potential issue with these, as with other, indices. The potential issues, described earlier in this discussion, relating to the use of total body length and bill width in body condition indices apply to RTFV 1 and RTFV 2. However, the effects on RTFV 1 of total body length possibly varying with body size are likely to be small given the large variation in tail fat volumes calculated in relation to a possible small variation in total body length. In addition to these, there is still some subjectivity in measuring fat area in the cross sectional images. In some images the exact border of the fat at the spine and/or the skin was not clear. Also, in some of the images a small area of the tail was not imaged, either because it extended beyond the edge of the probe or because of poor contact. In these cases, subjective assessments were made as to the position of the edge of the fat. However, the areas involved were small in comparison to the main part of the tail fat and the error was likely to be small. In addition to this, the assumption made in deriving the formula for tail area, that the tail fat could be approximated by three volumes with a triangular cross-section, constant width and steadily decreasing height is likely to have introduced inaccuracy into the tail fat volumes calculated.

A further point to RTFV 1 and RTFV 2 is the cost of the ultrasound unit and power supply requirements (in the case of the Aloka Prosound 2® AC 240 volt power was required so a car battery and inverter were used). It is unlikely that most projects would use this system in the field. However, as technology advances it is likely that similar units powered by rechargeable batteries will be available at a lower cost.

Relative Fat Depth 1 and Relative Fat Depth 2 (RFD 1 and RFD 2)

RFD 1 and RFD 2 are intended to give an objective assessment on a continuous scale of the amount of fat in a platypus' tail relative to its size. They are intended to be an improvement on TFI by assessing the amount of fat depth where ultrasonography indicates that there is the greatest variation in depth i.e. adjacent to the spine. Less importantly, the fact that higher values indicate a greater amount of tail fat relative to body size may be preferable to the situation with TVI, for which the reverse is true. The justification of using RFD 1 and RFD 2 as platypus body condition indices is that they correlate well with RTFV 1 and RTFV 2. They also use similar methods of data collection. As such, RFD 1 and RFD 2 suffer to some extent from the same possible limitations RTFV 1 and RTFV 2. In addition, RFD 2 correlated significantly with bill width in females and overall. RFD 1 correlated significantly in the overall population with both total body length and bill width. However, there were no significant correlations in male or females, so it is possible that this simply reflects a need to assess males and females separately.

The high correlation of mid-tail fat depth measurements using the two different ultrasound units leads to the possibility of assessing RFD 1 and RFD 2 relatively

cheaply and easily. The Signostics Speqview® is a relatively inexpensive, pocket sized, battery powered ultrasound unit. Figure 6.5 demonstrates that although the width of a platypus' tail cannot be assessed by sweeping the probe across the ventral aspect of the tail with the unit in M-mode, the images show that the position of the spinal cord and the depth of the fat adjacent to this can be measured. Such images could be obtained from a conscious or anaesthetised platypus in less than a minute.

Body Condition Index 1 and Body Condition Index 2 (BCI 1 and BCI 2)

BCI 1 and BCI 2 take a very different approach to assessment of body condition. They do not rely on a relationship between tail fat and body condition and they also intend not only to assess body fat but all body energy reserves. As described earlier in this discussion, this could in part be a reason for the fact that eight of the 36 correlations BCI 1 and BCI 2 and other body condition indices were not significant (Table 6.6). In the comparisons with LMs, BCI 2 performed poorly. The correlation of BCI 2 with bill length in females indicates that this body condition index may not be appropriate for assessing platypus body condition. As also described earlier, there are potential issues with the use of total body length as a basis for assessing body size as a result of the presence of fat at the end of the tail and the possibility of inaccurate measurement. Any error in total body length or tail length are likely to affect BCI 1 more than TFI, RTFV 1 or RFD 1 because body mass is likely to change much less than tail fat volume or cross-sectional area.

6.6 CONCLUSION

The following conclusions can be drawn from this investigation into platypus body condition indices:

- Further investigation into the relationship of tail fat to body fat is needed. Two possible methods are 1) further analysis of data from work with tritiated water at Lake Lea over 10 years ago (Munks *et al*., 2000), and 2) carcass analysis of roadkill platypuses.
- RTFV 1 and RTFV 2 are unlikely to be practical for most research projects.
- RFD 2 and BCI 2 showed correlation with bill width and as such are possibly not suitable body condition indices.
- BCI 1 is easily calculated from measurements routinely taken in most platypus research projects. Calculation of this index in future projects, and retrospectively for past projects, would help build up a large data set to assess whether seasonal changes in mean values are consistent with other body condition indices. The importance of a body condition index not relying on tail fat assessment will depend on the results of investigations into the relationship between tail fat and body fat.
- TVI has been used widely for over three decades. It is quick to assess. While there is significant overlap between categories when individual values are plotted against other body condition indices, at this stage it remains the base-line (as opposed to a gold standard) from which other body condition indices should be developed, and its continued use is recommended.
- RFD 1 is less subjective than TVI, is measured on a continuous scale and is well correlated with RTFV 1 and RTFV 2. Data for calculations can be gathered

quickly using a small, relatively inexpensive ultrasound unit. Use of this body condition index in future projects is recommended.

Chapter 7.

Health assessment of individual wild platypuses

7.1 INTRODUCTION

The health of individuals within wildlife populations can have important effects on reproductive success and longevity, and therefore population health. For example, infectious diseases have been involved to varying extents in species extinctions (Cunningham and Daszak, 1998; Daszak and Cunningham, 1999; Schloegel *et al.*, 2006). The health of individuals has also been shown to be an indicator of species decline (Munson and Karesh, 2002). However, in many situations there has been little information on individual health before species have been observed to be in decline.

A range of issues relating to individual platypus health have been addressed by previous studies. Platypus body mass, body size and body condition have commonly been reported (Grant and Temple-Smith, 1983; Serena and Williams, 1997; Connolly and Obendorf, 1998). Reference ranges for haematology and biochemistry parameters have been produced (Whittington and Grant, 1984; Booth and Connolly, 2008; Geraghty *et al.*, 2011). Tasmanian platypuses have consistently been reported to be approximately 50% heavier than those of mainland platypuses (Connolly and Obendorf, 1998; Munks *et al.*, 1998; Koch *et al.*, 2006; Gust and Griffiths, 2011). Body condition assessments have almost exclusively been made on the basis of an assessment of the amount of fat in the tail, and variations have been associated with food availability at different times of the year and energy use related to breeding (Temple-Smith, 1973; Grant and Carrick, 1978; Serena and Williams, 1997; Bethge, 2002).

Infection with, or exposure to, a range of infectious agents has also been reported (as described in Section 1.4). Mucormycosis is a disease of platypuses caused by the fungal organism *Mucor amphibiorum*, that most notably leads to granulomatous cutaneous

nodules and ulcers which can extend up to 10mm into the subcutaneous tissues (Munday and Peel, 1983; Obendorf *et al.*, 1993; Connolly *et al.*, 2000; Connolly, 2009). Granulomatous lesions have also been observed at necropsy in the internal organs of some individuals (Munday and Peel, 1983; Obendorf *et al.*, 1993). Although the lesions in some individuals have been observed to improve and it has been difficult to assess mortality rates, it is thought that mucormycosis can result in the death of individuals due to impaired thermoregulation (associated with loss of fur), impaired mobility, and susceptibility of affected animals to secondary infections and flystrike (Connolly and Obendorf, 1998; Munday *et al.*, 1998). Mucormycosis has been presumed to be the cause of death in platypuses with the disease found dead by members of the public (Obendorf *et al.*, 1993; Connolly *et al.*, 1998). Thirty percent prevalence has been observed in certain affected populations, and the infection is considered to be a conservation threat (Connolly *et al.*, 1998; Stewart, 2001).

Other infections show varying impacts on individual platypuses. Salmonellosis has been associated with morbidity and mortality in low numbers: one wild platypus, one established captive platypus and one platypus three weeks after entering captivity (Munday *et al.*, 1998). Serological evidence of exposure to *Leptospira* spp. has been found in approximately 50% of individuals in two separate studies and 66% in a third study, all in New South Wales (McColl and Whittington, 1985; Munday *et al.*, 1998; Loewenstein *et al.*, 2008). Whittington *et al.* (1990) found leptospires on histological examination of the kidneys of a platypus that had drowned in a fishing net. However, there have been no reports of clinical morbidity or mortality resulting from leptospirosis and the clinical effects of exposure to *Leptospira* spp. are unknown (Munday *et al.*, 1998; Loewenstein *et al.*, 2008). The death of a platypus associated with a cutaneous

granuloma caused by an unknown fungal agent, which was not *Mucor amphibiorum*, has been reported in Tasmania (Macgregor *et al.*, 2010). Papilloma virus has been reported as the likely cause of a high prevalence of papules on the webbing of the front feet of platypuses in the Healesville region of Victoria (Booth and Connolly, 2008). *Theileria ornithorhychi* has been reported to be present in the red blood cells of most platypuses (Collins 1986), but has only been associated with disease twice; both times in a juvenile platypus, from mainland Australia, with haemolytic anaemia that had a high level of infection (Booth and Connolly, 2008; Kessell *et al.*, 2014).

Despite this range of investigations into individual platypus health, no study has assessed all of these parameters in the same population to give an overall picture of individual health. The aim of this chapter was to gather baseline data on the health of individual platypuses in the Inglis River Catchment and the Seabrook Catchment and to look for individual, demographic, geographic and temporal patterns associated with health data results. An important part of this study was to investigate in detail any cases that showed clinical similarities to the disease mucormycosis.

7.2 MATERIALS AND METHODS

7.2.1 Study animals

Health examinations were performed on 130 wild platypuses (53 adult females, two juvenile females, 65 adult males, six subadult males, and four juvenile males) from the Inglis River catchment and 24 platypuses (10 adult females, one juvenile female, 11 adult males, and two juvenile males) from the Seabrook Creek catchment between 29/8/11 and 31/8/13. Repeat examinations were performed at the 12 recaptures (two

adult females at one recapture each, six adult males on one occasion each, and two adult males on at two recaptures each) that occurred during this period.

7.2.2 Individual health examinations and sampling

The following health assessment examinations were performed on all platypuses before anaesthesia:

- Visual assessment for alertness and external abnormalities.
- Body mass measurement (±10 g using digital Rapala® scales) weighing the platypus in its holding sack before anaesthesia and then subtracting the weight of the sack.

The following examinations were performed under anaesthesia:

- Sex and age by presence and morphology of spur using the method described by Temple-Smith (1973) and modified by Grant and Llewellyn (1991) and Williams *et al.* (2013). Males were classified into the following age categories: juvenile (<1 year of age; full spur sheath present January-June, full or partial spur sheath present July-December), subadult (12-18 months of age; partial spur sheath present January-June), adult (>15months; spur sheath absent). Females were classified into the following age categories: juvenile (<12 months; spur sheath retained March-December), subadult (spur sheath retained January-February) and adult (>9 months; no spur sheath retained). Note that because of variable times of spur sheath loss, there is overlap between the age categories. Williams *et al.* (2013) noted that it would be possible to misclassify a platypus age in a small proportion of non-adult individuals using this method alone. In

the present study, additional confidence in the results was provided by considering their body mass measurements.

- Tail Volume Index as described by Grant and Carrick (1978) (1 = very good, 5 = very poor).
- Moult class as described by Grant and Carrick (1978).
- Bill width (mm) at its widest point was assessed using vernier calipers.
- Total Body length (cm) using a tape measure (tip of bill to tip of tail, measured over dorsum).
- Tail length (mm) - distance from the tip of the tail (not including length of hair cover) to the caudal muscles of the body.
- Tail fat dimensions by ultrasound (as described in Section 6.3.2).

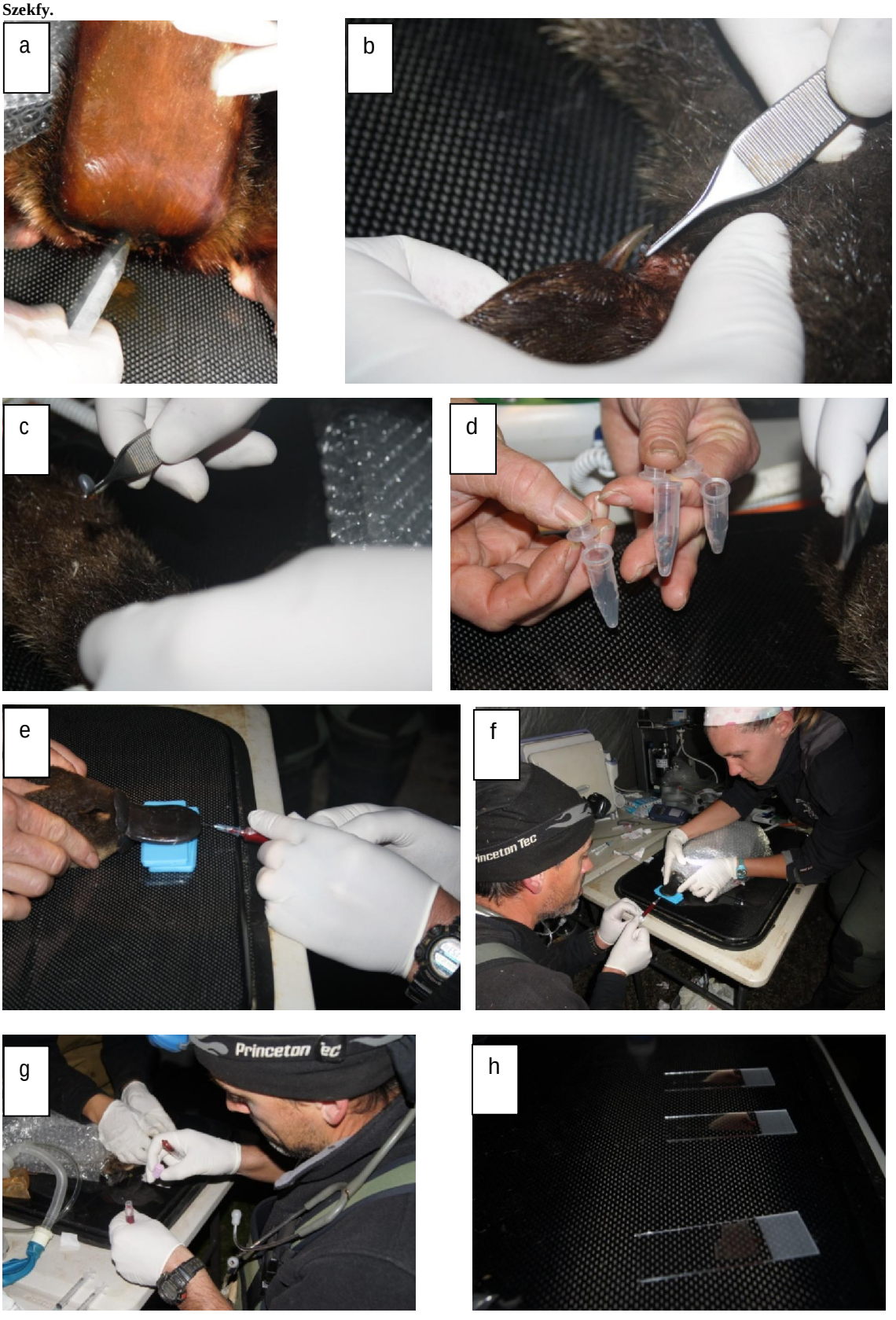
Relative Tail Fat Depth 1 (RFD 1) and Body Condition Index 1 (BCI 1) (see Chapter 6 for development of these parameters) were calculated as additional body condition indices.

The following biological samples were collected under anaesthesia:

- Ticks removed using forceps and placed in 70% ethanol, for identification (Figure 7.1).
- Excreta, up to 3ml collected by pipette via the cloaca for faecal flotation, and culture and sensitivity (Figure 7.1).
- Cloacal swab for microscopy, culture and sensitivity.
- Blood, up to 2ml collected using a 3ml syringe and 23G needle from the bill sinus, for biochemistry, haematology, microscopy and serology for *Leptospira* spp. and *Toxoplasma gondii* (Figure 7.1).
- Surface swabs from skin lesions for bacterial and fungal culture.

- Punch biopsies from skin nodules for histology, fungal and bacterial culture, and fungal PCR.

Figure 7.1. Biological sampling from platypuses (*Ornithorhynchus anatinus*) in the Inglis Catchment, Tasmania, a) collecting excreta sample from cloaca with a pipette, b) collecting ticks, c) collecting engorged tick, d) sampled ticks, e & f) collecting blood from venous sinus in the bill, g) transferring blood into sample tubes, and h) drying blood smears. **Photos: Geoff Dutton, David McArtor, David Maleca and Yolande Szekfy.**



7.2.3 Laboratory testing

Panfungal PCR was performed on a fungal culture isolate by SA Pathology, Women's and Children's Hospital, North Adelaide, South Australia. Panfungal PCR was also performed on shavings from paraffin embedded histology samples from two platypuses at Westmead Hospital Mycology Laboratory, Westmead, New South Wales. *Salmonella* typing was performed by the *Salmonella* Reference Laboratory, Microbiological Diagnostic Unit, Melbourne. All other laboratory testing was performed at the Department of Primary Industries, Parks, Water and Environment Tasmania (DPIPWE) Animal Health Laboratory, Mount Pleasant laboratories, Prospect, Tasmania. Packed cell volumes were determined manually (Graeme Knowles, personal communication) and other haematology parameters were determined using a Sysmex KX21N automated haematology analyser (Sysmex Corporation, Hyogo, Japan). All blood smear slides were reviewed for blood parasites and consistency with automated analyser results. A Konelab 20XTi (ThermoFisher Scientific) analyser was used to analyse sera for biochemical analytes. A faecal flotation test (using saturated magnesium sulphate) was conducted for parasitology on excreta where there was >1g of sample. Where there was insufficient sample for the floatation test, a wet preparation was used (Graeme Knowles, personal communication).

7.2.4 Haematology and biochemistry reference intervals

The process illustrated in Figure 7.2 was used to determine haematology and biochemistry reference intervals for adult platypuses; data from adult platypuses in the two study populations was combined for these analyses. Only the first set of data collected from each individual was used in this process. The process aimed to avoid possible influences of hyperthermia and the nasopharyngeal response that were

observed in some individuals under anaesthesia as described in Figure 7.2; to detect and describe seasonal variations in parameters where they occur; and to report results separately for males and females if statistically different. Data sets were examined and outliers removed as described in Figure 7.2. The process for determining the distribution of seasonally varying parameters involved assessing the correlation between the values for each parameter and a statistic for each day of the year (DOY) that, when represented graphically would form a sine wave with a period of one year. This correlation was repeated for varying sine wave positions until the best fit was found. To illustrate this, the process for determining the reference interval for Packed Cell Volume (PCV) in adult female platypuses is described in Appendix H.

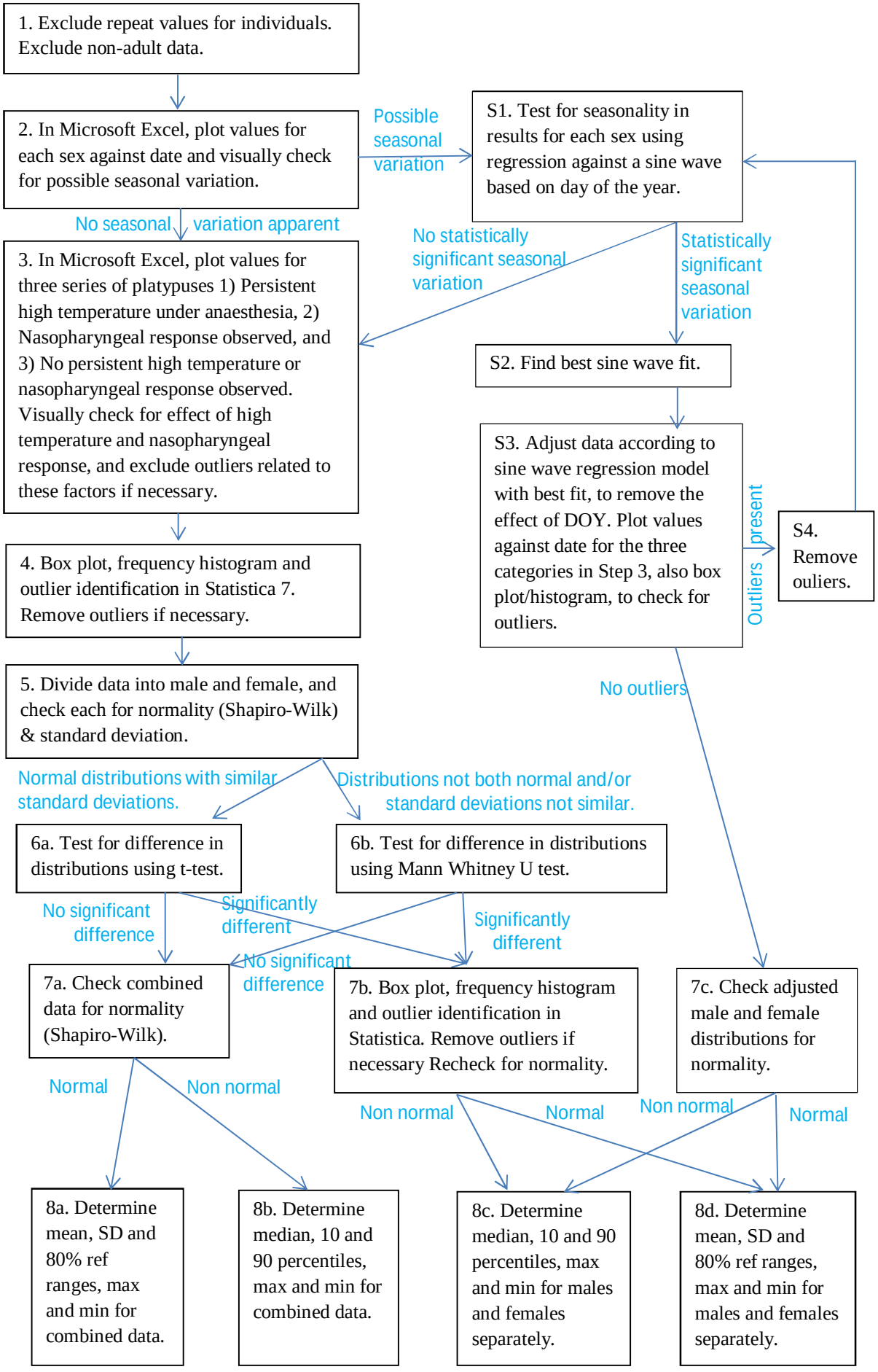
7.2.5 Statistical analysis

To investigate the possible effect of factors affecting morphometric data, separate mixed model ANOVAs for adult females and adult males were performed with body length, body mass, TVI, RFD 1 or BCI 1 as dependent factors, and season and subcatchment of capture as random factors. To further investigate the effects, forward stepwise regression was performed for each sex with each morphometric parameter (body length, body mass, TVI, RFD 1, BCI 1) as the dependent factor and time of year (spring/summer = September to February, autumn/winter = March to August), four variables chosen to reflect local habitat characteristics (altitude, amount of forest cover within a 500m radius, total water surface area within 500m of connected waterway, river length within 500m) and one variable chosen to represent the broader habitat characteristics of each subcatchment (proportion of forested land in the relevant subcatchment – including both native and plantation forest) as independent variables. To investigate the effects of factors that might affect haematology and biochemistry

parameters, forward stepwise regression was performed for each sex with each parameter (adjusted for sine wave variation where necessary to remove seasonal effects) as the dependent factor and area water within 500m, river length within 500m, forest area within a 500m radius, altitude and subcatchment forest area as independent variables. Statistical analyses were performed using Statistica 7 (Stat Soft Inc. Tulsa OK, USA).

A Mann Whitney U test was performed to investigate whether female serum calcium results showed from 31st January-28th May (n = 17, median = 2.28) were from a different distribution to those from 29th May - 30th January. Correlation coefficient (Pearson's r), and p value of correlations between seasonally varying haematology and biochemistry parameters and body condition indices, in females (F), males (M) and the overall sample population were calculated. To investigate possible causes of seasonal variation in certain red blood cell haematology parameters, the correlation coefficient, r, and p value of correlations between PCV, DOY sine wave parameter and individual/environmental temperature parameters, in female (F) and male (M) platypuses were calculated. To investigate possible causes of seasonal variation in certain serum biochemistry parameters, the correlation coefficient, r, and p value of correlations between albumin, DOY sine wave parameter and individual/environmental temperature parameters, in female (F) and male (M) platypuses were calculated.

Figure 7.2. Flow chart for reference interval development.



7.3 RESULTS

7.3.1 Morphometrics

The mean, standard deviation and range of values for morphometric data from each study catchment and subcatchment are listed in Table 7.1.

Table 7.1. Body length, mass and condition for adult females and males. (n), mean, standard deviation and range of values for body length, body mass and three measures of body condition (Tail volume index – TVI, Relative fat depth 1 – RFD 1, and Body condition index 1 - BCI 1). Values are (n) means ± 1 SD with range (min-max) shown.

	Females						Males					
	Body length (cm)											
Lower Inglis River	(6)	48.4	±	1.7	45.5	– 50.0	(3)	58.2	±	0.6	57.5	– 58.5
Flowerdale River	(14)	47.7	±	2	45.0	– 52.0	(17)	54.7	±	2.4	51.0	– 60.5
Inglis River	(7)	46.4	±	1.8	43.5	– 48.5	(6)	56.2	±	2.7	53.5	– 61.0
Blackfish Creek	(6)	47.3	±	1.2	46.0	– 49.0	(6)	53.8	±	2.7	51.0	– 58.5
Big Creek	(5)	46.8	±	1.2	45.0	– 48.0	(14)	55.5	±	3.1	52.0	– 63.5
Camp Creek	(8)	46.7	±	1.9	43.5	– 48.5	(12)	56.0	±	2.2	52.5	– 59.0
Upper Inglis	(6)	48.8	±	1.2	48.0	– 51.0	(6)	53.9	±	2.2	52.0	– 58.0
Garners Creek	(0)	-					(1)	52.0			52.0	52.0
Upper Flowerdale	(1)	45.0			45.0	– 45.0	(0)	-				
Overall Inglis	(53)	47.4	±	1.8	43.5	– 52.0	(65)	55.2	±	2.6	51.0	– 63.5
Seabrook Creek	(10)	47.5	±	1.8	45.0	– 50.5	(11)	54.6	±	2.4	51.5	– 58.5
Overall	(63)	47.4	±	1.8	43.5	– 52.0	(76)	55.1	±	2.6	51.0	– 63.5
	Body mass (kg)											
Lower Inglis River	(6)	1.43	±	0.09	1.33	– 1.57	(3)	2.45	±	0.17	2.34	– 2.65
Flowerdale River	(14)	1.32	±	0.18	1.10	– 1.63	(17)	2.01	±	0.31	1.54	– 2.71
Inglis River	(7)	1.27	±	0.18	0.98	– 1.52	(6)	2.20	±	0.29	1.88	– 2.70
Blackfish Creek	(6)	1.34	±	0.06	1.25	– 1.42	(6)	1.85	±	0.21	1.58	– 2.15
Big Creek	(5)	1.25	±	0.08	1.16	– 1.37	(14)	2.08	±	0.34	1.72	– 2.93
Camp Creek	(8)	1.27	±	0.12	1.08	– 1.42	(12)	2.14	±	0.27	1.63	– 2.47
Upper Inglis	(6)	1.38	±	0.15	1.22	– 1.63	(6)	1.96	±	0.25	1.69	– 2.41
Garners Creek	(0)	-					(1)	1.68			1.68	1.68
Upper Flowerdale	(1)	1.1			1.1	– 1.1	(0)	-				
Overall Inglis	(53)	1.32	±	0.15	0.98	– 1.63	(65)	2.06	±	0.31	1.54	– 2.93
Seabrook Creek	(10)	1.35	±	0.22	0.97	– 1.64	(11)	2.07	±	0.34	1.64	– 2.59
Overall	(63)	1.32	±	0.16	0.97	– 1.64	(76)	2.06	±	0.31	1.54	– 2.93

Table 7.1 (Cont'd). Body length, mass and condition for adult females and males. (n), mean, standard deviation and range of values for body length, body mass and three measures of body condition (Tail volume index - TVI, Relative fat depth 1 - RFD 1, and Body condition index 1 - BCI 1). Values are (n) means ± 1 SD with range (min-max) shown.

	Females						Males					
	TVI											
Lower Inglis River	(6)	3.2	± 0.8	2.0	–	4.0	(3)	3.0	± 1.0	4.0	–	2.0
Flowerdale River	(14)	3.1	± 0.8	2.0	–	4.0	(17)	3.1	± 0.9	4.0	–	2.0
Inglis River	(7)	3.4	± 1.1	2.0	–	5.0	(6)	3.2	± 1.0	4.0	–	2.0
Blackfish Creek	(6)	3.2	± 0.8	2.0	–	4.0	(6)	3.7	± 0.5	4.0	–	3.0
Big Creek	(5)	3.4	± 1.1	2.0	–	5.0	(14)	3.1	± 0.8	4.0	–	2.0
Camp Creek	(8)	3.5	± 0.9	2.0	–	5.0	(12)	3.1	± 1.0	5.0	–	2.0
Upper Inglis	(6)	2.7	± 0.5	2.0	–	3.0	(6)	2.8	± 1.2	4.0	–	1.0
Garners Creek	(0)						(1)	4.0		4.0		4.0
Upper Flowerdale	(1)	4.0		4.0	–	4.0					–	
Overall Inglis	(53)	3.2	± 0.8	2.0	–	5.0	(65)	3.2	± 0.9	5.0	–	1.0
Seabrook Creek	(10)	2.9	± 0.9	2.0	–	4.0	(11)	3.3	± 1.1	5.0	–	2.0
Overall	(63)	3.2	± 0.9	2.0	–	5.0	(76)	3.2	± 0.9	5.0	–	1.0
	RFD1											
Lower Inglis River	(3)	15.5	± 2.5	13.7	–	18.4	(2)	11.6	± 0.1	11.6	–	11.7
Flowerdale River	(10)	12.5	± 4.3	7.7	–	18.1	(11)	11.1	± 4.1	5.6	–	20.8
Inglis River	(6)	14.0	± 6.9	7.3	–	24.6	(5)	11.1	± 4.3	8.0	–	18.6
Blackfish Creek	(4)	15.9	± 5.5	8.5	–	21.2	(6)	10.6	± 3.5	5.0	–	13.8
Big Creek	(4)	12.0	± 5.2	6.3	–	18.3	(7)	10.6	± 2.5	6.4	–	12.9
Camp Creek	(6)	10.9	± 4.2	5.0	–	15.8	(10)	10.2	± 4.1	3.8	–	15.7
Upper Inglis	(6)	12.7	± 2.6	9.0	–	16.5	(6)	11.0	± 3.2	7.6	–	15.3
Garners Creek	(0)						(1)	8.0		8.0		8.0
Upper Flowerdale	(1)	10.4	N/A	10.4	–	10.4	(0)					
Overall Inglis	(40)	13.0	± 4.6	5.0	–	24.6	(48)	10.7	± 3.4	3.8	–	20.8
Seabrook Creek	(6)	13.7	± 3.8	8.9	–	19.5	(6)	11.0	± 5.0	5.9	–	20.3
Overall	(46)	13.1	± 4.4	5.0	–	24.6	(54)	10.8	± 3.6	3.8	–	20.8
	BCI 1											
Lower Inglis River	(6)	12.7	± 1.1	10.8	–	14.1	(3)	12.46	± 0.71	11.84	–	13.24
Flowerdale River	(14)	12.1	± 0.7	10.7	–	13.1	(17)	12.20	± 0.92	10.82	–	14.25
Inglis River	(7)	12.6	± 0.7	11.5	–	13.6	(6)	12.39	± 0.91	11.14	–	13.84
Blackfish Creek	(6)	12.7	± 1.1	11.3	–	14.4	(6)	11.84	± 1.00	10.74	–	13.57
Big Creek	(5)	12.2	± 1.1	11.1	–	13.7	(14)	12.12	± 0.63	11.12	–	13.04
Camp Creek	(8)	12.5	± 0.9	11.3	–	13.9	(12)	12.14	± 0.93	10.73	–	14.03
Upper Inglis	(6)	11.8	± 0.4	11.0	–	12.3	(6)	12.47	± 0.74	11.04	–	13.09
Garners Creek		-					(1)	11.95	±	11.95		11.95
Upper Flowerdale	(1)	12.3		12.3	–	12.3	(0)		±		–	
Overall Inglis	(53)	12.4	± 0.8	10.7	–	14.4	(65)	12.19	± 0.82	10.73	–	14.25
Seabrook Creek	(10)	12.6	± 1.3	10.6	–	14.0	(11)	12.65	± 0.76	11.58	–	13.91
Overall	(63)	12.4	± 0.9	10.6	–	14.4	(76)	12.26	± 0.82	10.73	–	14.25

7.3.2 Exposure to infectious agents

Single nodules were observed in the webbing of the front feet of five adult platypuses (four female and one male) and biopsy specimens were collected. The nodules were firm, roughly spherical in shape, ranged in size from ~3-7 mm in diameter with a slightly reddened surface appearance but no ulceration. The smallest nodule was considered to be a foreign body reaction on histology (Platypus 148). Another nodule had no evidence of fungal infection, but some spirochaetes were present on histology (Platypus 99; Figure 7.3). Three webbing nodules contained fungal elements on histology. One consisted of fungal hyphae only (Platypus 48; Figure 7.4), the other two contained fungal hyphae and spherules (Platypuses 125 and 95). Panfungal PCR performed on paraffin embedded shavings did not resolve the identity of the fungal organisms in one nodule containing fungal hyphae (Platypus 48) or one containing fungal hyphae and spherules (Platypus 95). However, *Phomopsis* sp. DNA was detected in the paraffin embedded shavings from the other nodule containing fungal hyphae and spherules (Platypus 125; Figure 7.5). A single fungal colony was cultured from a fresh specimen from this nodule. DNA sequencing identified this isolate as *Diaporthe* spp. Fungal culture was negative for the sample from Platypus 48, but was not performed on samples from Platypuses 95, 99 and 148 due to small biopsy size.

Results of investigations into exposure to infectious agents are summarized in Table 7.2, and the geographical location of the positive findings for infections/titres with prevalences of <90% are illustrated in Figures 7.6, 7.7 and 7.8.

Figure 7.3. Nodule in webbing of left fore foot of Platypus 99 - a) gross appearance, b) surgical scrub before biopsy, c) performing punch biopsy, d) removing biopsy from punch tool, demonstrating size of tissue sample. **Photos: David McArtor.**

Figure 7.4. Gross appearance of nodule in webbing of left fore foot of Platypus 48.

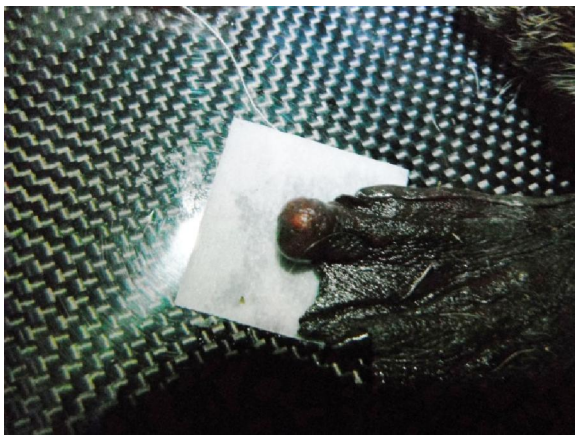


Figure 7.5 Nodule in webbing of right fore foot of Platypus 125, a) and b) gross appearance (note the patchy pigmentation which can be normal), c) punch biopsy of nodule, d), e) and f) Periodic Acid-Schiff stain of histological slides at increasing magnifications (scales not provided by laboratory). **Photos: Helen Robertson and Graeme Knowles.**

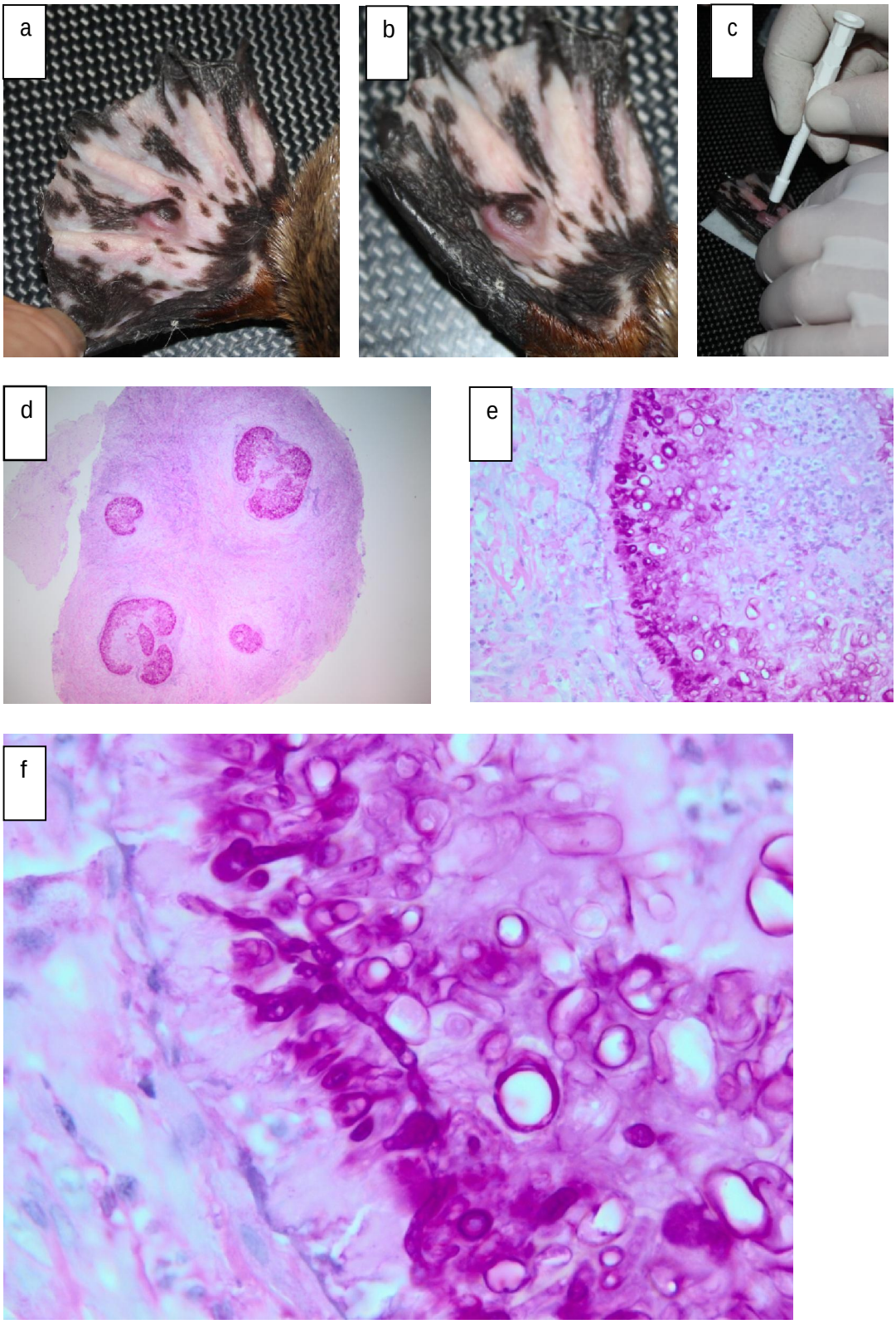


Table 7.2. Evidence of exposure to infectious diseases in platypuses – numbers in brackets indicate numbers tested in each age/sex category from which positive results were obtained.

Infectious agent	Testing method	n tested	Number adult +ves	No. SA* +ves	Number juvenile +ves	Total no. +ves	Observed Prevalence (%)	Confidence interval (%)
Salmonella spp.	Bacterial culture	151	2(62)♀ 5(75)♂	0(6)♂	0(3)♀ 0(5)♂	7	4.6	2.9 – 9.0
Leptospira spp.	Serology	115	4(49)♀ 7(57)♂	0(4)♂	0(2)♀ 0(3)♂	11	9.6	6.9 – 18.3
Fungal granuloma in foot webbing	Clinical exam, histology, culture, PCR	154	3(63)♀ 0(76)♂	0(6)♂	0(3)♀ 0(6)♂	3	1.9	0.8 – 3.8
Other granuloma in foot webbing	Clinical exam, histology, culture	154	1(63)♀ 1(76)♂	0(6)♂	0(3)♀ 0(6)♂	2	1.3	0.4 – 2.6
Mucormycosis	Clinical exam, histology, culture, PCR	154	0(63)♀ 1(76)♂	0(6)♂	0(3)♀ 0(6)♂	0	0	N/A
Theileria spp.	Microscopy on blood film	142	53(60)♀ 67(71)♂	5(5)♂	3(3)♀ 3(3)♂	131	92.3	90.1 – 99.4
Trypanosomes	Microscopy on blood film	143	52(60)♀ 68(71)♂	4(5)♂	3(3)♀ 4(4)♂	131	91.6	89.3 – 99.3
Toxoplasma gondii	Serology	110	1(48)♀ 0(53)♂	0(4)♂	0(2)♀ 0(3)♂	1	0.9	0.0 – 1.8
Cryptosporidia spp.	Excreta microscopy	112	0(52)♀ 1(54)♂	0(4)♂	0(1)♀ 0(1)♂	1	0.9	0.0 – 1.8
Coccidia spp.	Excreta microscopy	112	0(52)♀ 1(54)♂	0(4)♂	0(1)♀ 0(1)♂	6	5.4	3.3 – 10.5
Cestode ova	Excreta microscopy	112	0(52)♀ 1(54)♂	0(4)♂	0(1)♀ 0(1)♂	1	0.9	0.0 – 1.8
Ticks	Clinical examination	153	58(63)♀ 74(75) ♂	6(6)♂	3(3)♀ 6(6)♂	147	96.1	94.5 – 99.8
Leeches	Clinical examination	154	0(63)♀ 2(76)♂	0(6)♂	0(3)♀ 0(6)♂	2	1.2	0.3 – 2.4

***** SA = subadult

Figure 7.6. Locations of platypuses with webbing nodules, or from which a *Salmonella* spp. was isolated.

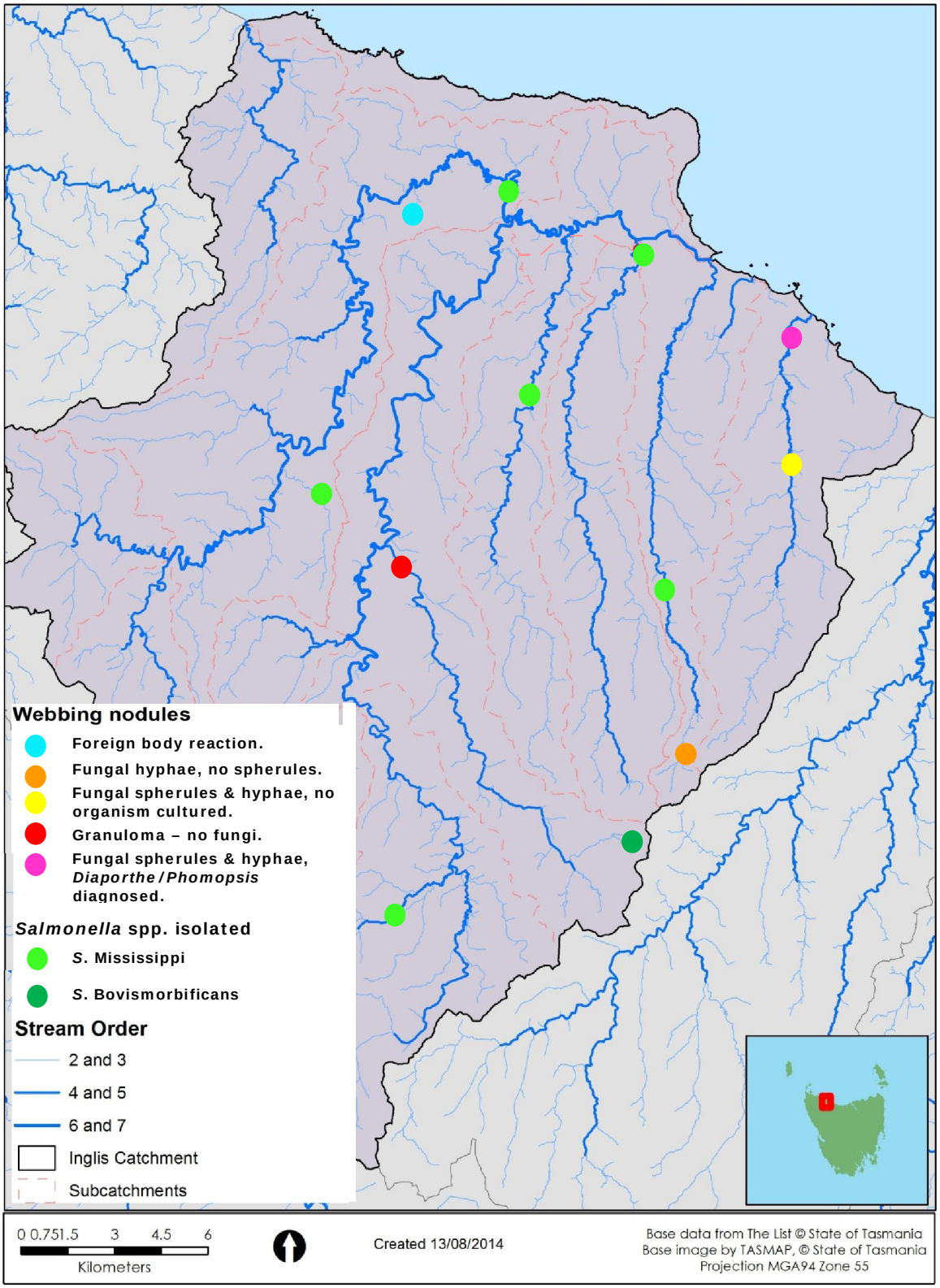


Figure 7.7. Locations of platypuses with serological titres to *Leptospira* spp. or *Toxoplasma gondii*.

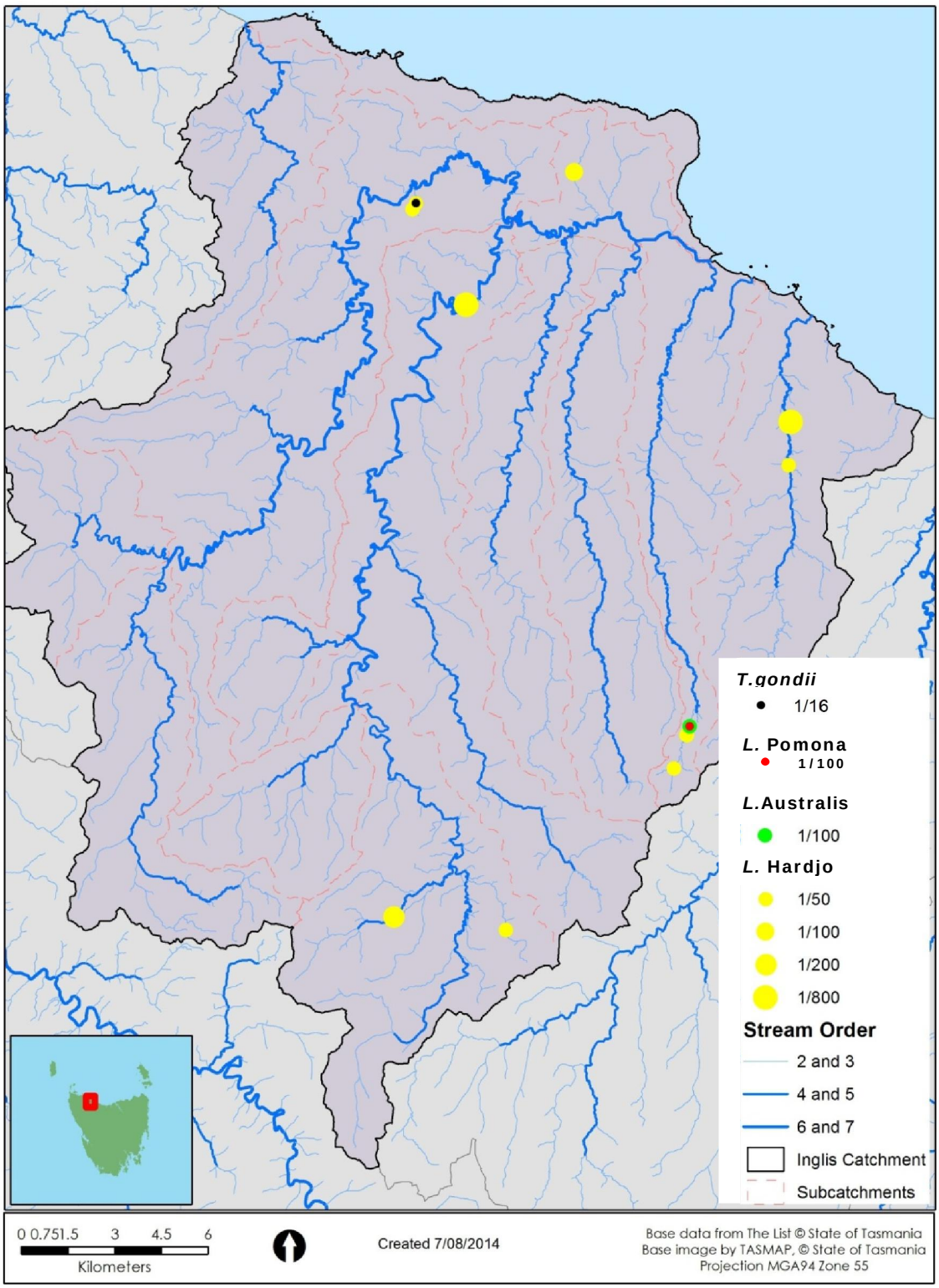
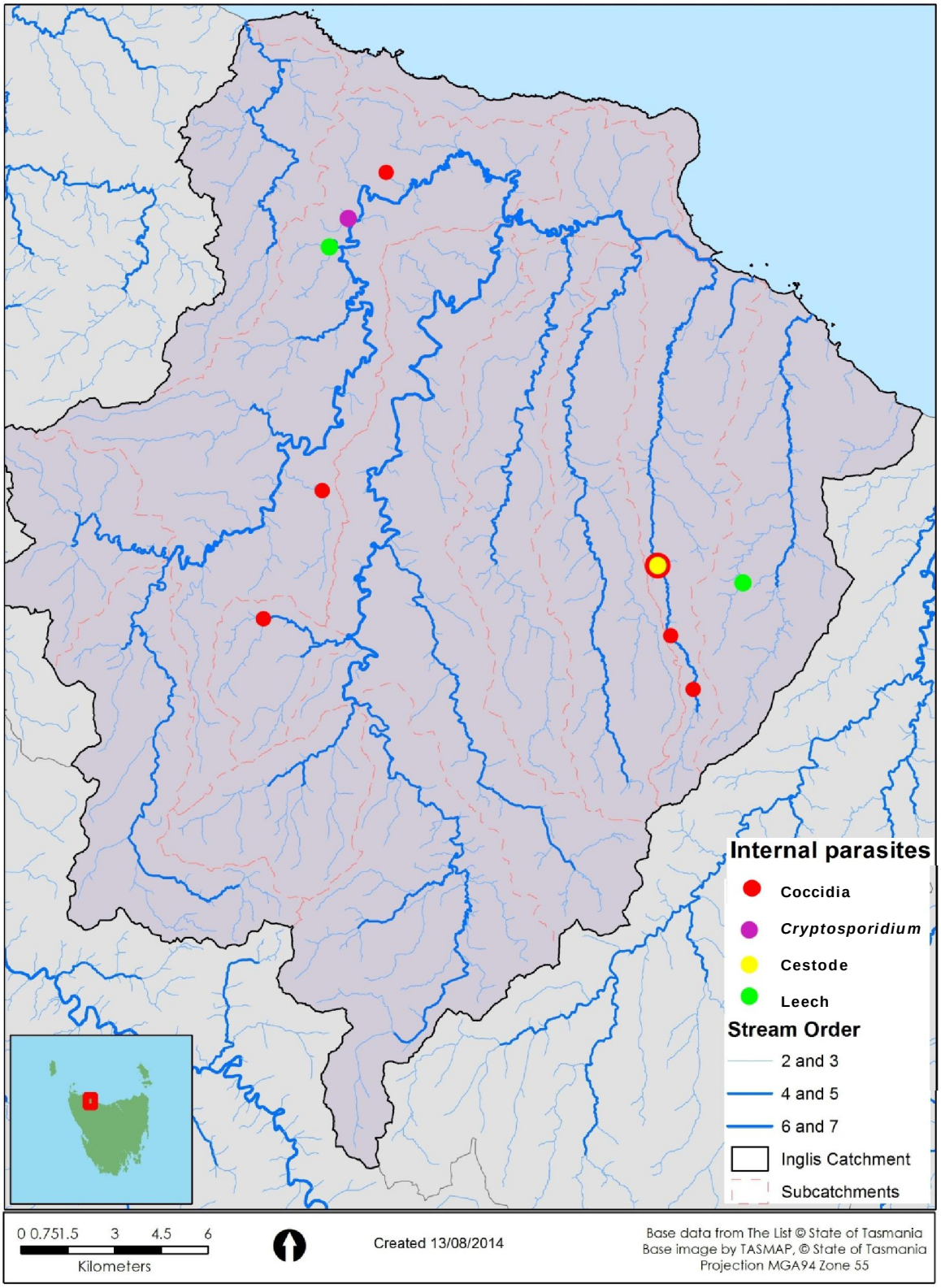


Figure 7.8. Locations of platypuses with parasites other than ticks.



7.3.3 Haematology and biochemistry reference intervals

The distributions of the observed haematology and biochemistry parameters are described in Table 7.3. For those parameters that varied seasonally, the distributions are illustrated in Figure 7.9. Seasonally varying parameters are listed separately for males and females. Non-seasonally varying parameters are listed separately for males and females where the distributions were found to differ significantly between the sexes. Non-marginal outlier haematology and biochemistry results that were excluded from the reference interval development process are shown in Table 7.4.

Table 7.3. Haematology and biochemistry reference intervals for adult platypuses (male and female combined where not specified).

			Non-parametric distributions					Normal distributions			
	Units	**n**	**Median**	**10th percentile**	**90th percentile**	**Min**	**Max**	**Mean**	**S.D.**	**Min**	**Max**
Haematology											
PCV ♀ *		57	46+(2.7*sine((DOY +202)*360/365))	42+(2.7*sine((DOY +202)*360/365))	49+(2.7*sine((DOY +202)*360/365))	37	53				
PCV ♂ *		66						46+(3.9*sine((DOY+235)*360/365))	3.16	36	55
RCC ♀ *	$x10^{12}$/L	57	9.49+(0.31*sine((DOY +174)*360/365))	8.5+(0.31*sine((DOY+174)*360/365))	10.24+(0.31*sine((DOY +174)*360/365))	7.27	11.21				
RCC ♂ *	$x10^{12}$/L	66	9.79+(0.87*sine((DOY +221)*360/365))	8.67+(0.87*sine((DOY +221)*360/365))	10.58+(0.87*sine((DOY +221)*360/365))	6.61	11.32				
Hb ♀ *	g/L	57	163+(9.34*sine((DOY +184)*360/365))	147+(9.34*sine((DOY +184)*360/365))	174+(9.34*sine((DOY+184)*360/365))	132	185				
Hb ♂ *	g/L	66						160+(14.5*sine((DOY +219)*360/365))	10.6	114	192
MCV	fL	126						47.8	2.7	40.0	54.5
MCH ♀	pg	58						16.9	0.8	15.1	18.6
MCH ♂	pg	68						16.4	0.8	13.6	18.0
MCHC ♀	g/L	58						353	11.7	329	384
MCHC ♂	g/L	68	346	323	365	290	435				
WCC	$x10^9$/L	124	25.3	15.0	41.3	8.30	60.80				
Neutrophils	$x10^9$/L	124	9.46	5.35	14.89	3.35	22.27				
Bands	$x10^9$/L	124	0.00	0.00	0.00	0.00	1.16				
Lymphocytes	$x10^9$/L	124	13.05	6.80	29.64	3.72	50.46				
Monocytes	$x10^9$/L	124	0.56	0.11	1.19	0.00	1.96				
Eosinophils ♀	$x10^9$/L	56	0.38	0.00	1.14	0.00	1.54				
Eosinophils ♂	$x10^9$/L	66	0.74	0.10	1.69	0.00	2.34				
Basophils	$x10^9$/L	124	0.00	0.00	0.00	0.00	0.41				
Total solids	g/L	125	72	66	78	61	88				
Fibrinogen	g/L	125	2	2	4	1	8				
Biochemistry											
CK F	U.I.	52	408.5	272	954	188	2346				
CK M	U.I	61	579	393	1247	276	2397				
AST	U.I.	109	681	477	915	419	1444				
ALP	U.I	104	102	71	153	47	202				
ALT	U.I.	107	269	184	424	123	542				
GLDH	U.I	106	7	44	189	35	373				
GGT ♀	U.I.	47						4.55	1.92	0	9
GGT ♂	U.I	57	4	1	5	0	7				
Total Bilirubin	µmol/L	104	2.7	1.6	5.2	1.1	9.8				
Cholesterol ♀	mmol/L	13	4.5	3.9	5.4	3.0	6.1				
Cholesterol ♂	mmol/L	13						4.53	0.79	3	6.1
Creatinine ♀	µmol/L	52	31	22	43	19	59				
Creatinine ♂	µmol/L	61						35	7.7	15	54
Urea	mmol/L	109	28.6	24.4	32.9	15.1	37.6				
Calcium ♀	mmol/L	48	2.46	2.09	3.77	1.88	4.22				
Calcium ♂	mmol/L	56						2.10	0.08	1.93	2.32
Magnesium ♀	mmol/L	48						1.04+(0.09*sine((DOY +205)*360/365))	0.12	0.61	1.33
Magnesium ♂ *	mmol/L	55						0.95+(0.12*sine((DOY +199)*360/365))	0.12	0.67	1.38
Phosphate ♀ *	mmol/L	49						1.96+(0.27*sine((DOY +135)*360/365))	0.35	1.16	2.93
Phosphate ♂ *	mmol/L	56						1.96+(0.19*sine((DOY +176)*360/365))	0.26	1.3	3.44
Na	mmol/L	110						151	2	145	157
K	mmol/L	110	2.30	1.50	3.30	1.20	4.90				
Na/K		110	67	46	100	30	132				
Chloride	mmol/L	108	108	106	111	103	114				
T protein	g/L	109						65.0	4.2	55.3	77.0
Albumin ♀ *	g/L	51						30.4+(1.5*sine((DOY +223)*360/365))	2.09	24.9	34.9
Albumin ♂ *	g/L	58						31.7+(1.2*sine((DOY +215)*360/365))	1.49	28.6	34.5
Globulin	g/L	108						34.0	3.5	27	44
A/G ratio ♀ *		51						0.90+(0.09*sine((DOY +176)*360/365))	0.11	0.65	1.18
A/G ratio ♂ *		57						0.95+(0.05*sine((DOY +236)*360/365))	0.10	0.75	1.27

Table 7.4. Non-marginal outlier haematology and biochemistry results.

Parameter and reference interval (10^{th} - 90^{th} percentile or 80% confidence interval)	Platypus number 43	122	57	78	84	19	125
AST (477-915) U.I.	1759	3017					
ALT (184-424) U.I.	860	1124					
GLDH(44-189) U.I.	542	1374					
GGT ♀ (2-7) U.I.				54	57		
GGT ♂ (1-5) U.I.			59				
Eosinophils ♀ (0-1.14) $x10^9$/L							3.68
Eosinophils ♂ (0.1-1.69) $x10^9$/L						3.62	

7.3.4 Patterns within health data

Separate mixed model ANOVAs for adult females and adult males with body length, body mass, TVI, RFD 1 or BCI 1 as dependent factors and season of capture and subcatchment of capture as random factors found no significant effects of subcatchment. Season was found to have a significant effect on body length in females ($p=0.009$) and on TVI in males ($p=0.001$). Forward stepwise regression for adult morphometrics (Table 7.5) showed a significant negative effect of subcatchment forest area on body mass in both sexes. In females, the significant positive effect on RFD 1, and negative effect on TVI, of the March to August time period suggested a seasonal effect on body condition.

Table 7.5. The results of forward stepwise regression testing the association between adult morphometrics with season and habitat characteristics.

	dependent factors	Independent factors tested (all) and significant relationships retained in the model (Beta and p values)					
	body length	Time of year†	Altitude	Forest cover[1]	Water area[2]	River length[3]	Subcatchment forest[4]
Males	total body length	-	-	-	-	-	-0.270 (p=0.024)
	body mass	-	-	-	-	-	-0.340 (p=0.004)
	TVI	-	-	-	-	-	-
	RFD 1	-	-	-	-	-	-
	BCI 1	-	-	-	-	-	-
Females	total body length	0.287 (p=0.038)	-	-	-	-	-
	body mass	-	-	-	-	-	-0.276 (p=0.037)
	TVI	-0.315 (p=0.025)	-	-	-	0.367 (p=0.012)	-
	RFD 1	0.344 (p=0.030)	-	0.454 (p=0.009)	-	-	-
	BCI 1	-	-	-	-	-	-

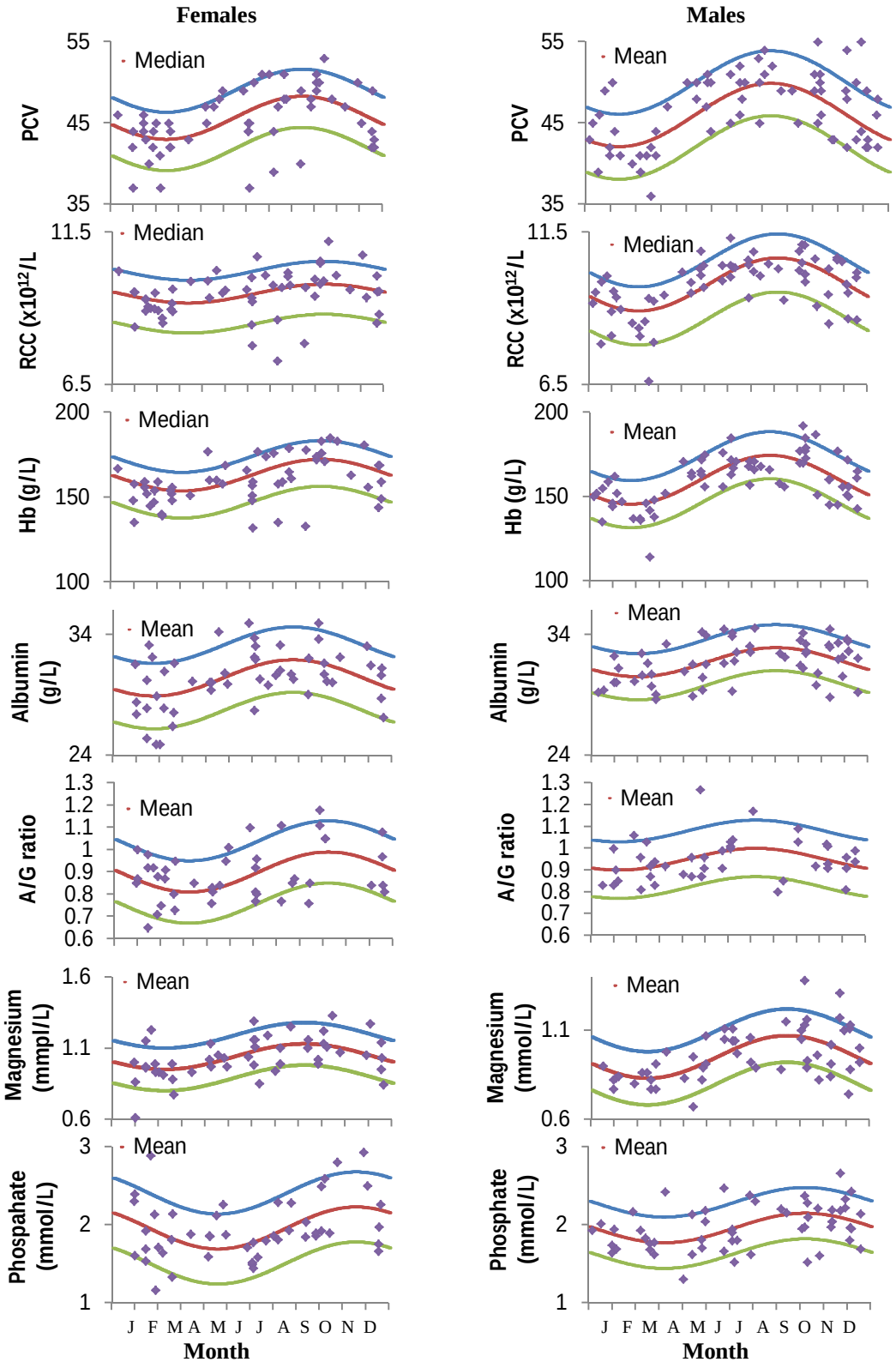
Independent factors not retained in the models are indicated with (-)
† September–February = 1, March–August = 0
[1] amount of forest cover within a 500 m radius
[2] total water surface area within 500 m of connected water
[3] river length within 500 m of connected water
[4] proportion of forested land in the relevant sub-catchment

Of the 25 platypuses with evidence of exposure to infectious agents other than external or blood parasites, nine were adult females and 16 were adult males. All of the individuals with evidence of fungal organisms within nodules in the webbing of a foot were females. One of these also had eosinophilia. The only individual to have a serological titre to *Toxoplasma gondii* also had a low titre (1:50) to *Leptospira interrogans* serovar Hardjo. Two individuals that were shedding coccidian-like oocysts also had other infectious agents in their excreta, one had *Salmonella* Mississippi and the other had a cestode-like ova. Another individual from which *Salmonella* Mississippi was cultured also had a low serological titre to *Leptospira interogans* serovar Hardjo. The only serological titres to *Leptospira interogans* serovar Pomona and *Leptospira interogans* serovar Australis were found in a single individual. The platypus from which

Phompsis spp./*Diaporthe* spp. were isolated also had a high eosinophil count (3.68 x 10^9/L).

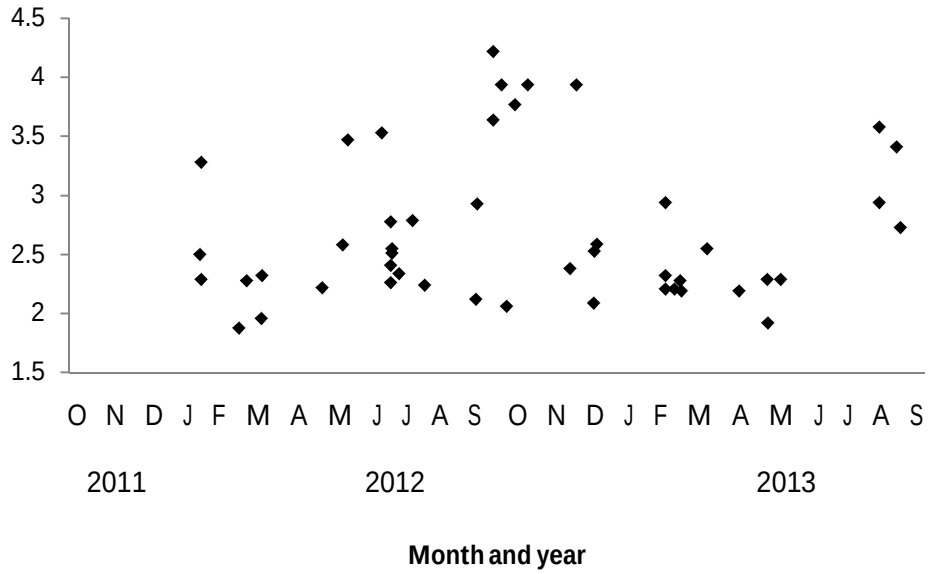
Seven parameters showed a significant correlation with a sign wave with a period of one year in both male and female platypuses. The reference intervals of these parameters as expressed by formulae based on DOY are illustrated in Figure 7.9. The observed values (with outliers removed) are also represented in Figure 7.9 to demonstrate how these relate to the reference intervals.

Figure 7.9. Graphical representations of reference intervals for parameters that showed seasonal variation. Red line = mean or median as labelled, blue line = 90 percentile or 1.28 x standard deviation above mean, green line = 10 percentile or 1.28 x standard deviation below mean, purple diamonds = observed values.



Values for serum calcium in males did not show seasonal variation. Values for serum calcium in females (Figure 7.10) did not fit the sine wave pattern of the parameters represented in Figure 7.9, however, a Mann Whitney U test showed that the distribution of results from 31st January to 28th May (n = 17, median = 2.28) had a significantly different distribution to those from 29th May to 30th January (n = 31, median = 2.73); U = 96, $p < 0.001$.

Figure 7.10. Serum calcium results of adult female platypuses plotted against month and year of capture.



Within individuals, red cell parameters were highly significantly correlated with each other ($p<0.001$), with $r > 0.77$ (Table 7.6). There were no significant positive correlations between red blood cell parameters and BCI 1 and RFD 1, or negative correlations with TVI. In males and the overall sample population, there was a low correlation between albumin and RCC. In Table 7.7 the correlation coefficient, r, and the p value for the correlations between PCV, DOY sine wave parameter, water temperature, nightly minimum temperature at Wynyard, platypus cloacal temperature at

induction, platypus cloacal temperature at recovery for male and female platypuses are summarised. Similarly in Table 7.8 the correlation coefficient, r, and the p value for the correlations between albumin, DOY sine wave parameter, water temperature, nightly minimum temperature at Wynyard, platypus cloacal temperature at induction, platypus cloacal temperature at recovery for male and female platypuses are listed. The results of forward stepwise regression testing the association between adult haematology and serum biochemistry parameters with habitat characteristics, are shown in Table 7.9. For calcium in females, forward stepwise regression also including time of year (Febrary-May = 1, June-January = 0) showed a significant effect of time of year (Beta = -0.474, p=0.001) and water area within 500m (Beta = -0.355, p=0.027).

Table 7.6. Correlation coefficient, (Pearson's r), and p value of correlations between seasonally varying haematology and biochemistry parameters and body condition indices, in females (F), males (M) and the overall sample population.

	BCI 1 F	RFD 1 F	TVI F	PCV F	RCC F	Hb F	Albumin F
PCV F	-0.097	0.027	-0.071				
	p=0.561	p=0.871	p=0.671				
RCC F	-0.144	0.011	-0.107	**0.741**			
	p=0.389	p=0.950	p=0.522	**p<0.001**			
Hb F	-0.118	-0.051	0.034	**0.926**	**0.788**		
	p=0.482	p=0.761	p=0.841	**p<0.001**	**p<0.001**		
Albumin	**-0.321**	**-0.362**	0.049	0.087	0.088	0.097	
F	**p=0.050**	**p=0.026**	p=0.769	p=0.604	p=0.601	p=0.562	

	BCI 1 M	RFD 1 M	TVI M	PCV M	RCC M	Hb M	Albumin M
PCV M	0.106	0.170	-0.233				
	p=0.506	p=0.281	p=0.137				
RCC M	0.017	0.056	-0.037	**0.810**			
	p=0.915	p=0.724	p=0.814	**p<0.001**			
Hb M	0.090	0.204	-0.147	**0.789**	**0.902**		
	p=0.570	p=0.195	p=0.354	**p<0.001**	**p<0.001**		
Albumin	0.218	0.023	0.001	0.248	**0.306**	0.196	
M	p=0.166	p=0.887	p=0.996	p=0.113	**p=0.049**	p=0.214	

Table 7.6 (Cont'd).

	BCI 1	RFD 1	TVI	PCV	RCC	Hb	Albumin
PCV	0.004	0.060	-0.161				
	p=0.972	p=0.600	p=0.155				
RCC	-0.055	-0.018	-0.059	**0.7754**			
	p=0.629	p=0.873	p=0.606	**p<0.001**			
Hb	0.008	0.073	-0.076	**0.830**	**0.859**		
	p=0.943	p=0.520	p=0.503	**p<0.001**	**p<0.001**		
Albumin	-0.106	**-0.278**	0.023	0.194	**0.249**	0.149	
	p=0.350	**p=0.013**	p=0.838	p=0.085	**p=0.026**	p=0.186	

Table 7.7. Correlation coefficient, r, and p value of correlations between PCV, DOY sine wave parameter and temperature parameters, in female (F) and male (M) platypuses.

F n=44	PCV	Induction temperature	Recovery temperature	Water temperature	Minimum air temperature	DOY sine wave value
Induction temperature	-0.200 p=0.193					
Recovery temperature	-0.285 p=0.061	**0.815** **p=<0.001**				
Water temperature	**-0.306** **p=0.044**	**0.571** **p<0.001**	**0.643** **p<0.001**			
Minimum air temperature	-0.151 p=0.327	**0.501** **p=.001**	**0.579** **p<0.001**	**0.796** **p<0.001**		
DOY sine wave value	**0.473** **p=0.001**	-0.260 p=0.088	**-0.358** **p=0.017**	**-0.737** **p<0.001**	**-0.613** **p<0.001**	

M n=44	PCV	Induction temperature	Recovery temperature	Water temperature	Minimum air temperature	DOY sine wave value
Induction temperature	-0.061 p=0.707					
Recovery temperature	-0.043 p=0.792	**0.818** **p<0.001**				
Water temperature	**-0.630** **p<0.001**	0.183 p=0.252	0.280 p=0.076			
Minimum air temperature	-0.402 p=0.009	0.204 p=0.202	**0.328** **p=0.036**	**0.707** **p<0.001**		
DOY sine wave value	**0.618** **p<0.001**	-0.170 p=0.290	-0.244 p=0.125	**-0.894** **p<0.001**	**-0.679** **p<0.001**	

Table 7.8. Correlation coefficient, r, and p value of correlations between albumin, DOY sine wave parameter and temperature parameters, in female (F) and male (M) platypuses.

F n=40	Albumin	Induction temperature	Recovery temperature	Water temperature	Minimum air temperature	DOY sine wave value
Induction temperature	-0.164 p=0.311					
Recovery temperature	-0.047 p=0.772	**0.855** **p<0.001**				
Water temperature	**-0.437** **p=0.005**	**0.575** **p<0.001**	**0.622** **p<0.001**			
Minimum air temperature	**-0.422** **p=0.007**	**0.543** **p<0.001**	**0.595** **p<0.001**	**0.819** **p<0.001**		
DOY sine wave value	**0.498** **p=0.001**	**-0.396** **p=0.011**	**-0.454** **p=0.003**	**-0.861** **p<0.001**	**-0.742** **p<0.001**	

M n=36	Albumin	Induction temperature	Recovery temperature	Water temperature	Minimum air temperature	DOY sine wave value
Induction temperature	0.244 p=0.151					
Recovery temperature	0.217 p=0.205	**0.905** **p<0.001**				
Water temperature	**-0.487** **p=0.003**	0.273 p=0.107	0.248 p=0.146			
Minimum air temperature	**-0.342** **p=0.041**	**0.361** **p=0.031**	**0.359** **p=0.031**	**0.775** **p<0.001**		
DOY sine wave value	**0.443** **p=0.007**	-0.146 p=0.394	-0.132 p=0.444	**-0.741** **p<0.001**	**-0.620** **p<0.001**	

Table 7.9. The results of forward stepwise regression testing the association between adult haematology and serum biochemistry parameters with habitat characteristics.

	Dependent factors	Independent factors tested (all) and significant relationships retained in the model (Beta and p values)				
		Altitude	Forest cover[1]	Water area[2]	River length[3]	Subcatchment forest[4]
Males	PCV	-	-	-	-	0.250 (p=0.049)
	RCC	-	-	-	-	-
	Haemoglobin	-	-	-	-	0.253 (p=0.045)
	MCV	-	-	-	-	-
	MCH	-	-	-	-	-
	MCHC	-	-	-	-	-
	WCC	-	-0.272 (p=0.043)	-	-	-
	Neutrophils	-	-0.282 (p=0.046)	-	-	-
	Lymphocytes	-	-	-	-0.311 (p=0.032)	-
	Monocytes	-	-	-	-	-
	Eosinophils	-	-	-	-	-0.342 (p=0.014)
	Basophils	-	-	-	-	-
	Bands					
	Total solids	-	-	-	-	-
	CK	-	-	-	-	-
	AST	-	-	-	0.375 (p=0.008)	-
	ALP	-	-	-	-	0.330 (p=0.0170)
	ALT	0.253 (p=0.046)	0.275 (p=0.027)	-	0.434 (p=0.001)	-
	GLDH	-	-	-	-	-
	GGT	-	-	-	-	-
	Total Bilirubin	-	-	-	-	-
	Cholesterol	-	-	-	-	-
	Creatinine		-	-	-	-
	Urea	-	-	-	-0.290 (p=0.031)	-
	Calcium	-	-	-0.355 (p=0.027)	-	-
	Magnesium	-	-	-		-
	Phosphate	-		-	-	-
	Sodium	-	-	-	-	-
	Potassium	-	-	-	-0.265 (p=0.044)	-0.263 (p=0.045)
	Na/K ratio	-	-	-	-	0.290 (p=0.028)
	Chloride	-	-	-	0.338 (p=0.024)	-
	Protein	-	-	-	0.425 (p=0.006)	-
	Albumin	-	-	-	0.332 (p=0.035)	-
	Globulin	-	-	-	0.339 (p=0.027)	-
	A/G ratio	-	-	-	-	-

Independent factors not retained in the models are indicated with (-)

[1] amount of forest cover within a 500 m radius

[2] total water area within 500 m of connected water

[3] river length within 500 m of connected water

[4] proportion of forested land in the sub-catchment

Table 7.9 (Cont'd). The results of forward stepwise regression testing the association between adult haematology and serum biochemistry parameters with habitat characteristics.

	Dependent factors	Independent factors tested (all) and significant relationships retained in the model (Beta and p values)				
		Altitude	Forest cover[1]	Water area[2]	River length[3]	Subcatchment forest[4]
Females	PCV	-	-	-0.390 (p=0.008)	-0.602 (p=<0.001)	-
	RCC	-	-	-0.309 (p=0.048)	-0.383 (p=0.015)	-
	Haemoglobin	-	-	-0.374 (p=0.016)	-0.446 (p=0.008)	-
	MCV	-	-	-	-0.394 (p=0.028)	0.366 (p=0.043
	MCH	-	-	-	-	0.437 (p=0.018)
	MCHC	-	-	-	0.432 (p=0.002)	-
	WCC	-	-	-	-	-
	Neutrophils	-	-	-	0.356 (p=0.026)	-0.492 (p=0.001)
	Lymphocytes	-	-	-	-	
	Monocytes	-	-	-	-	-
	Eosinophils	-	-	-	-	-
	Basophils	-	-	-	-	-
	Bands					
	Total solids	-	-	-	-	-
	CK	0.370 (p=0.012)	-	-	-	-
	AST	-	-	-	-	0.328 (p=0.049)
	ALP	-	-	-	-	-
	ALT	-	-	-0.275 (p=0.049)		-
	GLDH	-		-	-	-
	GGT	-	-	0.375 (p=0.22)	-	-
	Total Bilirubin	-	-	-	-	-
	Cholesterol	-	-	-	-	-
	Creatinine	-	-	-	-	-
	Urea	0.329 (p=0.035)	-	-	-	-
	Magnesium	0.389 (p=0.009)	-	-	-	-0.327 (p=0.027)
	Phosphate	-	-	-	-	-
	Sodium	-	-	-	-	-
	Potassium	-	-	-	-	-
	Na/K ratio	-		-	-	-
	Chloride	-	-	-	-	-
	Protein	-	-	-	-	-
	Albumin	-	-	-	-	-
	Globulin	0.439 (p=0.003)	-	-	-	-
	A/G ratio	-	-	-	-	-

Independent factors not retained in the models are indicated with (-)

[1] amount of forest cover within a 500 m radius

[2] total water area within 500 m of connected water

[3] river length within 500 m of connected water

[4] proportion of forested land in the sub-catchment

7.4 DISCUSSION

This chapter revealed possible seasonal effects on platypus body condition and possible habitat effects on body size. More than 90% of captured platypuses were infected with ticks, *theilleria* spp. and trypanosomes. Evidence of exposure to other infections, including *Salmonella* spp., *Leptospira* spp., and intestinal parasites, was low (<10%). Three platypuses had single fungal granulomas in the webbing of a fore foot, but no evidence of mucormycosis was found in any of the study animals. Strong evidence was found for seasonal variation in seven haematology/serum biochemistry paraemeters (PCV, RCC, Hb, albumin, A/G ratio, magnesium and phosphorus); and reference intervals for these were expressed as sine wave functions with minima occurring February-April in males and March-May in females. This chapter provides important reference data not only for each health parameter but also for the combined set of parameters in a single population.

It is well documented that platypuses are sexually dimorphic in body length and body mass (Grant and Temple-Smith, 1983; Connolly and Obendorf, 1998; Bethge, 2002; Gust and Griffiths, 2011). It is also well documented that platypuses in Tasmania are generally larger than those in mainland Australia (Connolly and Obendorf, 1998; Munks *et al.*, 1998; Koch *et al.*, 2006). The findings of this study are consistent with these observations. Studies of Tasmanian platypuses have reported mean mass in different river systems in the ranges 0.91-1.65kg and 1.47-2.5 kg for females and males, respectively (Connolly and Obendorf, 1998; Otley *et al*., 2000; Stewart, 2001; Bethge, 2002; Koch *et al.*, 2006; Olssen-Herrin, 2009; Gust and Griffiths, 2011). Mean body mass results in this study were close to the middle of these ranges and were very close to the mean values of 1.3 kg for females and 2.1 kg for males reported by Macgregor

(2008) in the Inglis River Catchment. Gust and Griffiths (2011) graphically represented mean body length values for platypuses from different river systems as 42-48 cm for females and 48-56 cm for males. The results of this study were close to the upper ends of these ranges and were higher than the values of 43cm and 49cm reported by Macgregor (2008) in the Inglis River Catchment. This finding may be a result of the prevention of hunching or lateral curving of the spine, associated with the use of anaesthesia in this study, which might otherwise reduce the measured body length. There were no obvious outliers for male and female morphometric data in the different subcatchments and the mixed model ANOVAs found no significant effects of subcatchment of capture. The results of forward stepwise regression shown in Table 7.6 suggest a negative relationship between the proportion of land that is forested in a particular subcatchment and body size may exist in both sexes of platypus. The possibility of such a relationship requires further investigation, but superficially appears to have similarities to the negative relationship (discussed at more length in Section 2.4) between capture numbers and forest area within 500m of sites (Section 2.3.5). In addition, the forward stepwise regression models provided additional evidence of seasonal change in body condition. The regression model for female body length showed seasonal effect. Figure 6.12 showed trimonthly mean values for female body length to vary by up to ~3cm. It seems likely that variations of this magnitude are at least in part a stochastic effect due to relatively small sample sizes rather than being entirely a result of increased tail length due to increased fat deposition at the tail tip in individuals in good condition as discussed in Section 6.4.

The observed prevalences of *Theileria*, trypanosomes and tick infections were high (>90%). The observed prevalences of other infections and serological titres were low

(<10%). It was decided that these prevalence values and the absence of apparent geographical, seasonal or age/sex category patterns in the distributions of positive platypuses made statistical analysis of the disease data inappropriate.

The isolation of *Salmonella* spp. from wildlife is not uncommon and prevalences similar to or higher than the 4.6 ± 1.7% observed in this study have been reported for a range of avian, reptilian and mammalian species (Quessy and Messier, 1992; Handeland *et al.*, 2002; Renter *et al.*, 2006; Phalen *et al.*, 2010; Scheelings *et al.*, 2011). Six of the seven isolates in this study were identified as *Salmonella* Mississippi (Edwards *et al.*, 1943). On average, this serovar is isolated from approximately 17 people per 100,000 in Tasmania annually (Ashbolt and Kirk, 2006). Approximately 80% of the human infections in Australia occur in Tasmania and its occurrence is thought to be associated with exposure to native animals and drinking untreated water (Ashbolt and Kirk, 2006). During the course of this project, an outbreak in people involving 11 confirmed and an estimated 25 suspected, cases of *Salmonella* serovar Mississippi occurred in Tasmania associated with eating at a particular hotel/restaurant over a three day period (OzFoodNet, 2014). Isolation of *Salmonella* Mississippi has previously been reported from wildlife species such as skinks, snakes, quolls, Tasmanian devils and kangaroos (Ball, 1992; Obendorf, 1993). This serovar has not previously been reported in platypuses. However, given the reported associations with wildlife species and water, it is not a surprising finding. *Salmonella* Bovismorbificans, the other serovar isolated in this project, is relatively common in Australia (Liesegang *et al.*, 2002). In the two years to 31st March 2014, *Salmonella* Bovismorbificans was isolated from 172 animals in Australia, consisting of 144 cattle, five dogs, four pigs, four sheep, three cats, two horses, one bird and 9 individuals of other species - including one platypus from this

study (Animal Heatlh Australia, 2012; Animal Health Australia, 2013; Animal Health Australia, 2014). This serovar has been reported to have been isolated from unspecified wildlife species in the Western Australian mainland and island populations (Iveson *et al.*, 2013). Approximately 100 human cases of *Salmonella* Bovismorbificans were reported annually in Australia between 1997 and 1999 (Stafford *et al.*, 2002). Outbreaks of human gastrointestinal disease due to *Salmonella* Bovismorbificans have been associated with raw vegetables in Europe and Australia (Liesegang *et al.*, 2002; Stafford *et al.*, 2002) and this has led to speculation that the bacteria could survive in soil and watery habitats leading to infection via vegetables. Transmission of *Salmonella* spp. to wildlife species from farmed cattle and pigs has been reported (Skov *et al.*, 2008). *Salmonella* Bovismorbificans has been isolated from cattle egrets (*Bubulcus ibis*) in Texas USA, but whether there was transmission between this species and domesticated animals was not determined (Phalen *et al.*, 2010). The association in Australia of this serovar with cows, and the possibility that it survives well in aquatic environments would appear to make platypuses in the two study populations, which inhabit an area where pasture for grazing cattle is a common land use, at risk of infection. However, further study would be required to determine the relationship. *Salmonella* spp. are common in wild animals and infections are usually subclinical (Uhart *et al.*, 2011). Consistent with this there were no indications of clinical disease in the salmonella-positive platypuses in this study. However, there is potential for disease in infected animals if they become stressed by other factors (Uhart *et al.*, 2011). In addition, from a fieldworker safety viewpoint, the 4.6% *Salmonella* spp. prevalence of observed in this study should be considered and incorporated into hazard reduction plans for future research projects that involve handling platypuses.

The prevalence of serological titres to *Leptospira* (9.6%) was low compared to previous studies in platypuses. Ten individuals had antibodies to *Leptospira interogans* var. Hardjo (*L.* Hardjo), and one had antibodies to both *Leptospira interogans* var. Pomona (*L.* Pomona) and *Leptospira interogans* var Australis. Seroprevalences to *L.* Hardjo of 47%, approximately 50% and 66% have been reported in three studies of platypuses in mainland populations (McColl and Whittington, 1985; Munday *et al.*, 1998; Loewenstein *et al.*, 2008). Between August 2004 and August 2014 there were 245 cattle submissions (some involving multiple animals) for *L.* Hardjo and/or *L.* Pomona to the Animal Health Laboratory in Launceston (Graeme Knowles, personal communication). Of these, 72 had at least one seroconversion to *L.* Hardjo and/or *L.* Pomona (Graeme Knowles, personal communication). Submissions ranged annually from 18 to 35 cases, each of which could consist of more than one animal. The numbers of cases with at least one animal that seroconverted to *L.* Pomona or *L.* Hardjo varied from 20 to 30 % annually (Graeme Knowles, personal communication). These findings suggest *L.* Pomona or *L.* Hardjo are widespread in the Tasmanian cattle population (Graeme Knowles, personal communication). The reason for the low prevalence in platypuses in this study is not clear. The two possible explanations are a low exposure to infection or a high mortality rate. Whilst McColl (1983) observed mild chronic interstitial nephritis, that could be consistent with *Leptospira* infection, during five of 20 platypus necropsies, *Leptospira* spp. are not known to cause clinical disease in platypuses (Munday *et al.*, 1998). However, further remote monitoring with in-stream microchip readers as described in Chapter 3 may help evaluate the survivorship of individuals with and without titres for *Leptospira* antibodies.

The five individuals with nodules in the webbing of the fore feet are an interesting series of case studies. Histology showed that fungal organisms were the cause of three of these lesions and one was a foreign body reaction. The last lesion was a granulomatous reaction but, although organisms with spirochaete morphology were seen on histology, the cause of the lesion was not conclusively determined. There were no obvious signs that these lesions were significantly affecting the platypuses and these cases are probably more significant in their similarity to the disease mucormycosis than they are as a cause of poor individual health. Lesions of mucormycosis have been reported on the webbing of the fore feet, and while this disease most notably can lead to large cutaneous ulcers, cutaneous nodules up to 10 mm in diameter have been reported (Connolly *et al.*, 2000). Histologically, mucormycosis lesions have been described as granulomas or lesions with granulomatous/pyogranulomatous inflammation containing spherules characteristic of *M. amphibiorum* infection (Connolly *et al.*, 2000). Daughter spherules (single, 11.3±2.5 µm in diameter) and mother spherules (18.0±5.8 µm in diameter and containing a mean of 4.7±3.2 daughter spherules) have been reported (Connolly *et al.*, 2000). Pyogranulomatous dermatitis containing fungal mother spherules of 15-20 µm in diameter, and fungal hyphae were identified histologically in nodules from Platypuses 125 and 95. The lesion from Platypus 48 has similar histological characteristics, including the presence of fungal hyphae, however spherules were absent. Panfungal PCR on paraffin embedded tissue samples excluded *Mucor* spp. infection as a possible diagnosis in all three of these cases. Fungi were detected in samples from Platypuses 48 and 95 but identification was unresolved, probably as a result of the presence of more than one species. *Phomopsis* spp. DNA was detected by PCR in paraffin embedded tissue samples from Platypus 125, and the isolate cultured from samples from the same lesion was identified as *Diaporthe* spp. *Phomopsis* and

Diaporthe are names used to describe the same genus of fungus (Udayanga *et al.*, 2011). This duplication in nomenclature arises from the occurrence in this (and many other) fungal species of a telomorph and anamorph reproductive form (Udayanga *et al.*, 2011). Organisms in this genus can lead to disease in a wide range of plant hosts. They have also been reported as the cause of subcutaneous infections in the fingers of two immunosuppressed people most likely as a result of accidental inoculation of the organism into subcutaneous tissues by pricking with plant material (Sutton *et al.*, 1999; Garcia-Reyne *et al.*, 2011). The ability of certain fungi to change between unicellular (spherules) and multicellular filamentous (hyphae) forms in response to environmental changes is known as fungal dimorphism (Nadal *et al.*, 2008). It has been identified to occur commonly in a range species including certain plant pathogens, six human pathogens and *Mucor* spp. (Connolly *et al.*, 2000; Stewart and Munday, 2005; Klein and Tebbets, 2007; Nadal *et al.*, 2008). Understanding of the triggers for this process and the species capable of undergoing it is incomplete (Nadal *et al.*, 2008; Garcia-Reyne *et al.*, 2011). Fungal spherules and hyphae have been reported in a human case of keratitis caused by an organism of the *Phoma* genus which is morphologically similar to *Phomopsis*/*Diaporthe* (Rishi and Font, 2003; De Gruyter *et al.*, 2009; Udayanga *et al.*, 2011). The findings of this study suggest that *Phomopsis* spp. should be added to the list of dimorphic fungi. In addition to this, the absence of *Mucor* spp. in the lesions from Platypuses 125 and 95, have implications for the diagnosis of mucormycosis. Both these lesions were clinically consistent with lesions of mucormycosis and histologically both contained spherules that have been described as characteristic of mucormycosis (Connolly *et al.*, 2000). Connolly *et al.* (2001) described the diagnosis of mucormycosis based on the gross appearance of lesions, the presence of spherules in fine needle aspirates and histological sections and culture of *M. amphibiorum*. Connolly (2009)

stated that suggestive lesions and the culture of *M. amphibiorum* are required for a diagnosis of mucormycosis to be made, and that further support for this diagnosis can be provided by the presence of spherules on wet or histological sections and/or detection of antibodies to *M. amphibiorum* by ELISA. However, Gust *et al.* (2009) diagnosed the disease on the basis of clinical signs only. Public sightings of the disease were described as possible cases by the same authors (Gust *et al.*, 2009). Of more concern, at least one case in northwest Tasmania has been described as a confirmed case when the original report described it as a "reliable sighting" (Munday *et al.*, 1998; Gust *et al.*, 2009). The only detailed reports of mucormycosis based on the use of fungal culture with mating experiments, histology, and/or cytology describe cases in waterways and water catchment areas which ultimately drain into the Tamar River in northern Tasmania (Munday and Peel, 1983; Obendorf *et al.*, 1993; Connolly *et al.*, 1998; Munday *et al.*, 1998; Stewart, 2001). Despite this, it has been suggested that the disease has spread to catchments west of the Tamar basin (Munday *et al.*, 1998; Gust *et al.*, 2009). Macgregor *et al.* (2010) suggested that caution needed to be exercised in diagnosis of mucormycosis, particularly in river catchments where the disease has not been previously diagnosed. The findings of this study reinforce this suggestion and also indicate that the finding of spherules in fine needle aspirates or impression smears may lead to false positives if not confirmed by histology, fungal culture and/or PCR.

The introduction of foreign material, fungal organisms and possibly spiral bacteria during foraging on the floor of water bodies seems to be a likely cause of the webbing nodules observed. Further research is required to determine whether Platypuses 125 and 95, who were both captured in the Seabrook Creek Catchment and whose lesions both contained fungal spherules and hyphae, reflect one off cases or a low but consistent

prevalence of infection with a particular *Phomopsis/Diaporthe* species. The observed zero prevalence of mucormycosis is encouraging for the two populations, as mucormycosis is considered a conservation threat (Munday *et al.*, 1998). It also suggests that data from this project would make a suitable comparison for a future study using similar methods to investigate the effects of mucormycosis on the health of infected populations.

The high prevalence of *Theileria*, trypanosomes and ticks is consistent with the findings of previous studies, and the suggestion that, with the exception of two reports of juveniles with *Theileria*, these organisms do not usually lead to significant disease in platypuses (Munday *et al.*, 1998; Booth and Connolly, 2008; Kessell *et al.*, 2014). Blood parasite burdens were not quantified in detail in this project, however, in associated projects, Paparini *et al.* (2013) and Paparini *et al.* (2014) used genomic studies to, respectively, identify two piroplasm genotypes (possibly representing two species previously thought to be the single species *Theileria ornithorhynchi)*, and four closely related genotypes of *Trypanosoma binneyi*. The prevalence of coccidia, cryptosporidium, antibodies to *Toxoplasma gondii*, cestodes and leeches were low and unlikely to be having an effect at a population level in this study area. Evidence of coccidia and *Toxoplasma gondii* in platypuses have been previously reported but, although cestode plerocercoids in the lungs of platypuses have been recorded, this is the first report of cestode ova in the excreta of a platypus (McColl, 1983; Whittington, 1988; Whittington *et al.*, 1992). This is also the first report of leeches on platypuses and, in an associated study, Paparini *et al.* (2014) provided evidence that leeches may have originally transmitted *T. binneyi* from fish to platypuses.

The reference intervals determined in this study for biochemistry and haematology parameters provide a baseline for platypuses examined under isoflurane anaesthesia. Geraghty *et al.* (2011) and Booth and Connolly (2008) reported reference intervals for samples taken from similar numbers of conscious platypuses from Tasmania and Victoria, respectively. Although these studies reported results differently to this study and to each other, comparisons can be made. It should be noted that many of the 95% confidence intervals reported by Booth and Connolly (2008) seem too narrow to have been derived from distributions with the listed maximum and minimum. For instance, the upper and lower confidence limits for PCV were 49 and 51, respectively, and the minimum and maximum values were 35 and 62, respectively. However, deductions can be made by relating the ranges to the mean/median values observed in this study and by comparing minimum and maximum values. It should also be noted that there is a transposition of the values for GGT and AST in Table 6 of Geraghty *et al.* (2011). In general, the previously reported ranges appear to have been derived from parameter distributions similar to those in this study. The exceptions to this, however, are the red blood cell parameters PCV, RCC and Hb. The distribution of observed values for these parameters in this study (illustrated in Figure 7.9 without any adjustment for seasonal variation) appear to have a lower mean/median and narrower range than those reported by Geraghty *et al.* (2011) and Booth and Connolly (2008). For comparison, the minimum and maximum values for PCV observed by Booth and Connolly (2008) were 35 and 62, respectively, and the unadjusted 2.5 and 97.5 percentiles reported by Geraghty *et al.* (2011) were 42 and 64.8, respectively. The two most likely causes for this are 1) differences in sample handling, storage and laboratory testing, and 2) differences in observed values between samples taken from conscious platypuses and those taken from platypuses anaesthetised with isoflurane. Although the results for

PCV, RCC and Hb from a male and a female that underwent a nasopharyngeal response and one hyperthermic male were excluded as outliers on the upper end of the observed values, these possible complications during anaesthesia did not appear to have a major effect on the results. However, isoflurane has been shown to lead to decreased PCV, RCC and Hb results in ferrets, ponies and rats (Taylor, 1991; Marini *et al.*, 1997; Deckardt *et al.*, 2007) and this may have occurred in the platypuses. In ferrets, this has been shown to be due to splenic sequestration of red blood cells (Marini *et al.*, 1997). The stress of handling, which may be higher during sampling in non-anaesthetised platypuses, has been shown to lead to increases in these red blood cell parameters due to splenic contraction in other species (Raskin, 2009; Harvey, 2011). It is not currently possible to determine whether the values obtained for these red blood cell parameters for platypuses sampled conscious or under isoflurane anaesthesia are a better reflection of the animals before capture. However, it appears that the values are different for platypuses sampled in these two situations and they should be interpreted in the light of appropriate reference intervals. Isoflurane anaesthesia was used in this study and is advocated to reduce stress associated with physical restraint required for blood sample collection and other time-consuming or stressful procedures.

Of the seven platypuses with outlier haematology or biochemistry values listed in Table 7.4, clinical disease was found in only one animal. Platypus 125 had a fungal granulomatous dermatitis (nodule) in the webbing of a forefoot (see Section 7.3.4) and an eosinophil count above the reference interval. Another individual (Platypus 19) also had an elevated eosinophil count but the animal appeared clinically normal and there were no other biochemical or haematological findings to explain the raised eosinophil count. Two platypuses had AST, ALT and GLDH above the reference intervals and

three had GGT above the reference intervals. Because all five platypuses appeared clinically normal, these findings suggest but do not confirm, subclinical hepatopathies or cholangiohepatopathies, respectively (Graeme Knowles, personal communication).

The results of forward stepwise regression with biochemistry/haematology parameter as dependent variable and capture site habitat characteristics as independent variables show a number of significant effects but these do not form any interpretable patterns. A proportion of female platypuses were found to have elevated blood calcium levels between the end of May and the end of January when compared to males. Given the timing of the breeding season determined in Chapter 5, it seems likely that this variation is related to lactation. However, at this stage it is not possible to elucidate the mechanism by which it occurs. The sine wave appearance of the plots of observed values for PCV, RCC, Hb, Albumin, albumin/globulin ratio, magnesium and phosphate against date are striking findings. I am not aware of any other reference intervals that have been presented as functions of a sine wave. It is intended that this approach will allow more meaningful assessment of results and, while the seasonal functions in Table 7.3 are not simple, the graphical representation of reference curves in Figure 7.9 should allow easy interpretation and demonstrates that the shape and values of the reference curves provide an appropriate description of the observed data.

The changes in albumin/globulin ratio, magnesium and phosphate likely reflect seasonal changes in albumin; magnesium and phosphate being in part bound to albumin in the blood (De Swiet, 2002; Randell *et al.*, 2008). Reduced values for PCV, RCC, Hb and albumin have been observed during hyponutrition (Greig and Boynea, 1956; Artacho *et al.*, 2007). Decreases in PCV, RCC and Hb have also been observed during lactation

(EI-Nouty and AI-Haidary, 1990). Climatic conditions and physiological rhythms have also been suggested as causes of seasonal changes in these parameters (Sealander, 1964; DelGiudice *et al.*, 1992; Collazos *et al.*, 1998; Woods and Hellgren, 2003).

The reference curve minimum values determined in this study for the seven seasonally varying haematology/biochemistry parameters were in March/April for females and February/March in males, with the exception of phosphate values for which minima occurred approximately one month later. Mean daily air temperatures and mean monthly rainfall for Wynyard also follow seasonal patterns (Climate data online, 2014). The highest mean temperatures and lowest mean monthly rainfall for all years measured have occurred in February (Climate data online, 2014). The lowest mean temperatures and highest mean monthly rainfall for all years measured have occurred in July (Climate data online, 2014). Bobbi *et al.* (2003) reported water temperature data recorded at three sites in the Inglis River Catchment over three years. The graphical representation of these data showed a sine wave pattern of seasonal variation similar to that of the seasonally varying haematology/biochemistry parameters, with maximum values in February and minimum values in August/September (Bobbi *et al.*, 2003). In this study, the first cloacal temperature taken after induction of anaesthesia showed a similar sine wave pattern to those of the seven seasonally varying haematology/biochemistry parameters (Appendix B). This pattern was much reduced for the last cloacal temperature before recovery of anaesthesia (Appendix B).

These observations suggest a link between climate and the seven seasonally varying haematology/biochemistry parameters. Such a link could arise 1) as an artefact caused by variation in body temperature of platypuses during fieldwork, 2) as a result of

variation in food availability or 3) as a result of variations in metabolic demands. Tables 7.7 and 7.8 demonstrate multiple correlations between platypus body temperature, ambient temperatures and DOY sine wave parameter, demonstrating the likely interrelatedness of these variables. Tables 7.7 and 7.8 do not show any significant correlations between PCV/albumin and first/last cloacal temperature during anaesthesia. This suggests that the observed variations of the seven haematology/biochemisty parameters was not related to body temperature at or shortly before sampling and is unlikely to be an artefact caused by capture/holding/handling. However, Table 7.7 shows that PCV correlated significantly with water temperature and DOY sine wave parameter, and Table 7.8 shows that albumin correlated significantly with water temperature, minimum air temperature and DOY sine wave parameter. These results suggest the seasonally varying parameters vary with ambient temperature in recent hours/days or over a longer period of time.

The timings of reference curve minima (with the exception of phosphate in males) are close to the timings of mean minimum body condition indicated by investigations in Chapter 6. In five of the seven seasonal parameters (PCV, RCC, Hb, A:G ratio, phosphate), the fluctuations in the results for females lagged behind those of males by approximately one month. In the other two parameters (albumin and magnesium) the male fluctuations lagged behind those of females by 6-8 days. As mentioned above, low PCV, RCC, Hb and albumin have been observed at times of negative energy/protein balance, that could also lead to low body condition (Greig and Boynea, 1956; EI-Nouty and AI-Haidary, 1990; Artacho *et al.*, 2007). The timing of the reference curve minima in this study and the fact that they largely occur later in females may indicate that the demands of the breeding season and lactation have an effect on these fluctuations.

However, Table 7.6 demonstrates that positive correlations between these seasonal parameters and measures of body condition were not present in this study. Although this may be a result of the limitations for the indices used to assess body condition as discussed in Chapter 6, it suggests that the observed seasonal variation in haematology/biochemistry parameters did not primarily reflect seasonal changes in food availability or physiological energy demands.

Keatinge *et al.* (1984), Yahav *et al.* (1997) and Sutherland *et al.* (1958) reported significant changes in PCV in response to changes in ambient temperature in euthermic humans, chickens and rabbits, respectively. Keatinge *et al.* (1984) exposed people to six hours of surface cooling in moving air at 24°C. Minimal changes in PCV were observed after one hour of cooling, but a significant increase (mean 7%) in PCV occurred after six hours (Keatinge *et al.*, 1984). Yahav *et al.* (1997) exposed broiler chickens to a range of constant (10-35°C) and fluctuating ambient temperatures for three weeks. Haematocrit (a parameter equal to or very closely related to PCV) was observed to be increased in chickens exposed to low temperatures (Yahav *et al.*, 1997). Sutherland *et al.* (1958) exposed rabbits to temperatures of 4°C or -15°C for periods of one to 10 weeks. Haematocrit values were observed to be increased after eight and 10 weeks of cold exposure, but not after one week (Sutherland *et al.*, 1958). Increased PCV at low temperatures is considered to be a response to the need to deliver increased amounts of oxygen to tissues to support an increased metabolic rate for heat production (Keatinge *et al.*, 1984; Yahav *et al.*, 1997).

Platypuses are able to maintain their body temperature in air or water temperatures down to 5°C (Grant and Dawson, 1978a). Bethge (2002) reported a near doubling of

metabolic rate between a minimum metabolic rate in January and a maximum rate in July and, due to increased conductance of the fur when wet, it is water temperature that is the greatest determinant of daily metabolic rate (Grant and Dawson, 1978a; Grant and Dawson, 1978b). These findings in the platypus, and the reported link between PCV and increased metabolic rate in cold conditions in other species, are consistent with the correlations observed in this study between PCV and ambient (particularly water) temperatures.

Although increased PCV with cold exposure appears to be a consistent finding, varying changes in plasma proteins have been observed (Sutherland *et al.*, 1958; Yahav *et al.*, 1997), Sutherland *et al.* (1958) noted that, within the timeframe of their study, some haematology/biochemistry parameters other than PCV appeared to change quickly with cold exposure and then returned to values closer to normal. At least in the early stages of cold exposure, increased PCV levels may have been mediated by decreased blood volume as a result of decreased plasma albumin levels (Sutherland *et al.*, 1958; Yahav *et al.*, 1997). This mechanism would not be consistent with the similar variation over time in this study of red blood cell and albumin related parameters. However, in this study platypuses were not in an experimental situation, but were exposed to temperatures changing gradually over a period of months in the environment in which they have evolved. It seems possible that in this situation, elevated PCV levels in response to cold might be maintained by factors other than albumin mediated changes in blood volume, such as increased red blood cell production.

Artacho *et al.* (2007) described that red cell parameters can be indicators of physiological state but that their use can be limited by the scarcity of reference intervals,

natural variations in these parameters, and uncontrolled environmental conditions. Nevertheless, assessment of biochemistry and haematology results remain an important tool. Regardless of the cause (or causes) of the observed seasonal variations in seven parameters in this study, being aware of the potential for seasonal variation is important for assessing individual health. Given the large geographic range of distribution of the platypus and the variation of climate and breeding season timing within this range, it seems likely that the timing, magnitude and possibly the occurrence of these seasonal changes will vary between populations. The observations of this study suggest that it is important for platypus research projects reporting haematology and biochemistry parameters to look for seasonal changes in their data.

Chapter 8.

General discussion

8.1 POPULATION HEALTH IN THE INGLIS RIVER AND SEABROOK CREEK CATCHMENTS

This project has demonstrated the value of taking a holistic approach to conservation research, in the form of a population health assessment incorporating individual health, genomics and more traditional ecological factors. Baseline population health data, that for many species has been absent when population declines have occurred, was collected for platypuses. The study populations, which inhabit river catchments dominated by agriculture and forestry, showed few signs of poor population health or anthropogenic impacts. However, evidence that the most productive parts of the study area are more favourable both to platypuses and to agriculture demonstrates the importance for the species of including such areas in conservation management plans.

The results indicate that currently there is little evidence that the study populations are in poor health. Persistence of 80% of captured individuals at the sites of their captures during the course of the project, and 42% captured 4-5 years previously, combined with evidence of movement to other locations in four individuals during the course of this project does not suggest a high mortality rate (Chapters 2 and 3).

The lower diversity at the MHC class II DZB gene observed in the Seabrook Creek Catchment compared to some locations studied by Lillie *et al.* (2012), was consistent with the smaller size of the population in this catchment. Genetic diversity, however, was considerably higher than on King Island where the platypus population appears to be monomorphic at this locus (Lillie *et al.*, 2012). Alleles were generally well distributed through the Seabrook Creek Catchment (Chapter 4). Evidence was found that population density is higher in areas that have been cleared for agriculture. It was

likely that naturally higher productivity in these areas has led to a naturally higher carrying capacity for platypuses (Chapter 2).

Evidence of exposure to infectious disease was not higher than those in previous studies and no evidence of clinically significant disease caused by infections was found. Anthropogenic exposure to infectious agents appeared to be relatively low. Seroconversion to *Leptospira* spp. was at most 20% of those in previous studies, in which domestic animals have been considered the likely source of exposure. Only one individual (0.7%) was positive for a *Salmonella* strain likely to be related to domestic animals. Only one individual (0.9%) showed evidence of exposure to *Toxoplasma gondii*. Nodules were observed in the forefoot webbing of five platypuses (3.2%). Of these, three were determined to be fungal in origin, two contained fungal spherules (a histological feature previously considered only to occur in platypuses in cases of mucormycosis) and one was the result of the first recorded infection in a platypus by an organism in the *Phomopsis/Diaporthe* genus (Chapter 7).

Investigations into reproductive seasonality demonstrated that the platypus breeding season is later in Tasmania than on mainland Australia (Chapter 5). The use of in-stream microchip readers provided insights into platypus activity, including findings consistent with reproductive behaviour (Chapter 3).

Evidence was found that platypus PCV, RCC and Hb results are lower in samples taken under isoflurane anaesthesia than from conscious animals. Seasonal variation in eight haematology/biochemistry parameters was observed, and for seven of these the

distribution of results was best represented by reference curves that vary sinusoidally over the course of a year (Chapter 7).

8.2 RECOMMENDATIONS FOR PLATYPUS CONSERVATION MANAGEMENT PLANS

Four recommendations for platypus conservation management plans that arise from the findings of this study relate directly to human activities in and around platypus habitat or to monitoring approaches:

- Firstly, having determined the timing of the platypus breeding season in Tasmania it would be advisable to avoid activities such as water body dredging, dam building and riparian vegetation management between November and April, during which time juveniles are likely to be confined to the burrow and be most vulnerable to disturbance.
- Secondly, although evidence of anthropogenic exposure of platypuses to infectious agents was minimal, preventing access of stock to water bodies, also recommended to prevent bank erosion and increased water turbidity (Grant and Temple-Smith, 2003), could mitigate this further.
- Thirdly, the conclusion that the areas of the study catchments that have been cleared for agriculture are the areas that naturally have a higher platypus population density, suggests that protection of natural habitat in these more productive areas is important for platypus conservation. This is in contrast to the current approach globally which is largely based on residual protection, with areas reserved for conservation primarily being areas that are not in demand for other human activities (Margules and Pressey, 2000).

- The last recommendation for platypus conservation management plans is for the implementation of population health monitoring programs in both apparently stable and at risk populations. Grant (2012) suggested that monitoring may not be useful for assessing the impacts of human activities. However, in this study, the addition of investigation into exposure to infectious disease, genomic studies, the use of in-stream microchip readers to monitor survivorship and movements to more traditional ecological studies of distribution, age/sex population structure and habitat has provided a method of performing meaningful platypus conservation monitoring.

8.3 THE PLATYPUS POPULATION HEALTH ASSESSMENT FRAMEWORK AND FURTHER RECOMMENDATIONS FOR FUTURE RESEARCH

The Population Health Assessment Framework recommended for future research projects is presented in Table 8.1, along with contexts for interpretation and key inputs from this study. The inclusion of core measures/methods recommended for all studies, and non-core measures/methods, anticipates that the range of investigations undertaken may vary between projects according to resources, population specific factors and researcher interests or expertise. For instance, in locations with few streams suitable for the use of in-stream microchip readers the use of other remote monitoring techniques, such as acoustics tags (Griffiths *et al.*, 2013), may be appropriate. Equally, where particular land use practices are to be monitored, it may be appropriate to make more detailed habitat assessments than undertaken in this study. If the resources are available, the genomic aspect of this study could be expanded upon to include additional loci involved with the immune system, as well as loci without immune system function. Conversely, if funds or researcher experience required for abdominal ultrasonography

are not available, information on reproductive success could be gathered using oxytocin in females to assess the proportion of individuals that are lactating during the second half of the expected local lactation period.

Table 8.1. Platypus population health assessment framework and key inputs from this study. Core measures/methods in bold.

Field of study	Measures and methods	Interpretation and selected references	Key results and conclusions of this study
Demographics	**Distribution - public survey, live capture,** visual observation of water bodies.	Presence/absence in different water bodies/ sections of water bodies. Look for changes in distribution over time (Grant and Llewellyn, 1991; Connolly and Obendorf, 1998;Rohweder and Baverstock, 1999; Otley, 2001;Lunney *et al*.,2004; This study).	Platypus sightings and live captures broadly distributed through accessible areas of study populations (Chapter 2).
	Population age structure Adult sex ratio.	Age structure: consider effects of reproductive success, mortality rates of different age classes, capture methods (Grant, 2004; Gust and Griffiths, 2011; Serena and Williams, 2012; This study) Adult sex ratio: consider suitability of habitat for nesting burrows, and effects of capture methods and population decline. Consider effects of sex ratio on future population size (Grant, 2004; Gust and Griffiths, 2011; Serena and Williams, 2012; This study).	10% of captured individuals were non-adults – a low figure compared to other studies possibly due to the catchment-wide distribution of fieldwork sites, reduced adult mortality rates or other demographic differences. Overall adult sex ratio (F:M) of captured platypuses was 0.83:1. Further investigation is required to assess observed differences between subcatchments. Bias towards male captures could result from different use of habitat between the sexes (Chapter 2).
	Population density/size - public survey, capture rates/numbers ± remote monitoring, population size estimation.	Look for differences in public sightings and live capture numbers/rates between locations and over time (Grant and Llewellyn, 1991; Connolly and Obendorf, 1998;Rohweder and Baverstock, 1999; Otley, 2001;Lunney *et al*.,2004; Furlan *et al*., 2012; This study).	More platypuses captured in areas cleared for agriculture, suggesting a link with local productivity. Estimated density of 0.9platypuses/10,000km^2 similar to results of previous studies, but likely to be an underestimate. Baseline data on frequency of local public sightings (Chapter 2).
	Individual survivorship - In-stream microchip readers, live capture, acoustic tags.	Assess in relation to observed or potential threats to population (Grant, 2004; Williams *et al*., 2013; This study).	82% platypuses known to have remained alive during course of this study, 42% of individuals captured 4-6 years previously known to be still alive (Chapter 3).
	Movement/migration - instream microchip readers, live capture, radiotracking, acoustic tags	Assess habitat use and population stability (Serena, 1994; Gust and Handasyde, 1995; Serena et al., 1998; Otley 2000; Bethge 2003; Grant, 2004; Griffiths *et al*., 2013; Williams *et al*., 2013; Serena and Williams, 2013; This study).	80% of captured platypuses were resident to their capture area. Home ranges appear to be larger for females than males (Chapter 3).
	Timing and rates of reproduction - In-stream microchip readers, exam of cloacal swabs for sperm, endocrinology, detection of lactation after oxytocin injection, abdominal ultrasound.	Females – behavioural patterns, serum progesterone conc^n, proportion with ovarian structures/eggs in breeding season, proportion with milk 2-4 months after breeding season. Males – behavioural patterns, serum testosterone conc^n, testis size, proportion with large, active testes in breeding season (Temple-Smith, 1973Carrick and Hughes, 1978; Grant et al., 1983; McLachlan-Troup, 2007; Handasyde et al., 1992; Jakubowski et al., 1998; New et al., 1998; Morrow and Nicol, 2009; This study).	Reproductive endocrinology, changes is testis size, occurrence of ovarian follicles, detection of sperm on cloacal swabs and behavioural data suggest that the breeding season occurs in November/December in the study populations. Only 1 apparently adult male had a small testis in the breeding season. Over 2 years, 3 monitored females appeared to have 5 pregnancies successful to ~20 days after initial retirement to the burrow.

Table 8.1 (Cont'd). Platypus population health assessment framework and key inputs from this study. Core measures/methods in bold.

Field of study	Measures and methods	Interpretation and selected references	Key results and conclusions of this study
Individual Health	**Body size and body condtion (TVI, RFD 1 & BCI 1) morphometrics and tail ultrasound.**	Assess for geographical, habitat and seasonal effects on body size and body condition indices (Temple-Smith, 1973; Hulbert and Grant, 1983; Munks *et al.*, 2000; Handasyde *et al*., 2003: This study).	TVI and RFD 1 results suggested body condition higher in winter. Evidence that body condition was higher in areas with less surrounding forest cover, possibly related to local productivity (Chapter 7).
	Mucormycosis prevalence - clin exam, biopsy and microbiology of skin lesions.	Detailed investigations to assess presence/absence (±prevalence) of mucormycosis (Munday and Peel, 1983, Obendorf *et al*., 1993, Connolly *et al*., 1998, Stewart, 2001, Macgregor *et al*., 2010, This study).	0% observed prevalence in study populations. 3 platypuses observed with nodules in webbing of fore feet, 2 of which contained fungal spherules – mucormycosis ruled out, Phompsis/Diaporthe diagnosed in one case, likely opportunistic infection (Chapter 7).
	***Salmonella* and other GI bacterial infections - bacterial culture on excreta/cloacal swab.**	Interpret in relation to low prevalence and absence of associated clinical signs in this study (Munday *et al*., 1998; This study).	4.6% observed prevalence of *Salmonella* – 6 cases of S. Mississippi, 1 case of cow/water-associated S. Bovismorbificans (Chapter 7).
	***Leptospira* spp. serology.**	Interpret in relation to low seroprevalence in this study but 50%+ seroprevalence in previous studies (McColl and Whittington, 1985; Munday *et al*, 1998; Loewestein *et al*., 2008; This study).	9.6% observed prevalence of antibodies to *Leptospira* spp. Low in comparison to previous mainland studies despite cattle grazing being a major land use in study areas (Chapter 7).
	GI parasites - exam of excreta.	Interpret in relation to very low prevalence and absence of associated clinical signs in this study (Munday *et al*., 1998; This study).	Observed prevalences: Cryptospordia spp – 0.9%, Coccidia spp. – 5.4%, cestodes – 0.9%. No associated clinical signs observed (Chapter 7).
	External parasites - clinical exam.	Interpret in relation to high prevalence of ticks without serious disease in most studies (Munday *et al*., 1998; This study).	96.1% observed prevalence of ticks. First report of leech attachment – observed prevalence 1.2% (Chapter 7).
	Blood parasites - blood film exam.	Interpret in relation to high prevalence in most studies, but only 2 reports of associated disease (Collins et al., 1986; Munday *et al*., 1998; Kessel et al., 2014; Paparini et al., 2014; This study).	Observed prevalences: Theileria spp. – 92.3%, trypanosomes – 91.6%. No associated clinical signs observed (Chapter 7).
	***Toxoplasma gondii* - serology.**	Interpret in relation to low seroprevalence in this study, but disease in marsupials (Vogelnest & Woods, 2008; This study).	0.9% seroprevalence. No associated clinical signs seen (Chapter 7).
	Papilloma virus - exam and biopsy of skin lesions.	Assess presence/absence, ± prevalence and associated clinical signs (Booth and Connolly, 2008).	Not observed in study catchments.
	Haematology and serum biochemistry .	Assess for seasonal changes as well as associations with other aspects of individual health exams and environmental factors (Booth and Connolly, 2008; Geraghty et al., 2011; This study).	PCV, RCC and Hb values generally lower than previous studies, possibly associated with sampling during anaesthesia. Distributions of 3 haematology and 4 biochemistry parameters could be expressed as reference curves varying sinusoidally over 12 months (Chapter 7).

Table 8.1 (Cont'd). Platypus population health assessment framework and key inputs from this study. Core measures/methods in bold.

Field of study	Measures and methods	Interpretation and selected references	Key results and conclusions of this study
Genomics	**Diversity of immune system genes - MHC II,** other genes with immune function.	Consider effect of diversity on likely ability of population to respond to new infectious challenge (Acevedo-Whitehouse and Cunningham, 2006; Lillie *et al*, 2012; This study).	12 MHC II DZB gene alleles from 18 platypuses in Seabrook Creek Catchment. Alleles well distributed geographically. Intermediate heterozygosity in comparison to previous study populations likely related to population size (Chapter 4).
	Overall genetic diversity and gene flow - non immune system genes, microsatellites and mitochondrial DNA.	Consider effect of diversity of ability of population to respond to new environmental or anthropogenic challenges. Assess degree of isolation of the whole population or parts of it (Kolomyjec *et al*., 2009; Gongora *et al*., 2012; Furlan *et al*., 2012).	-
Environmental factors	**Broad habitat variables from GIS or other mapped data.**	Investigate effects on capture numbers/rates, body size/condition, and movement data (This study).	There were negative effects of local forest area on capture numbers (Chapter 2) and of subcatchment forest area on individual body size (Chapter 7), suggesting links with local productivity.
	Riparian habitat characteristics - detailed visual assessment.	Investigate effects on capture numbers/rates, body size/condition, and movement data (Rohweder, 1992; Woon, 1995; Grant and Bishop, 1998; Serena et al., 2001).	-
	Benthic macroinvertebrates - Environmental sampling.	Investigate effects on capture numbers/rates and body size/condition (Serena et al., 2001; McLachlan-Troup, 2007).	-
	Geology - mapped data.	Assess possible links between local productivity and both platypus population density and individual size by investigation correlations with local geology (Otley, 2001; Rohweder and Baverstock, 1999; This study).	-
	Stream morphology and hydrology - existing data or environmental monitoring.	Investigate effects on capture numbers/rates, body size/condition. Investigate effects of flood events on pre-emergence young (Serena and Williams, 2010; Serena et al., 2014; This study).	Anecdotal report of public sighting of dead juvenile platypuses after summer flooding event (Chapter 5).
	Man-made structures - mapped data or visual assessment.	Investigate effects on capture numbers/rates, movement and gene flow (Serena et al., 1998; Serena et al., 2010; Kolomyjec, 2010).	-
	Climate - field measurement or meteorological records	Investigate effects on haematology/serum biochemistry parameters and body condition (Temple-Smith, 1973; Munks *et al*., 2000; Handasyde *et al*., 2003; This study).	Seasonal variation in 3 haematology and 4 biochemistry parameters appeared to be influenced by seasonal climatic variation Chapter 7).

Specific findings of this study that warrant further investigation include seasonal variations in haematology/biochemistry parameters, the prevalence and nature of webbing nodules and the reliability of body condition indices. There is scope to use in-stream microchip readers to investigate a range of factors including long-term survivorship, habitat use, short-term and long-term movements, breeding behaviour and avoidance of fyke nets. It is also important that the baseline population health data provided by this project is built upon by performing similar research in populations with different characteristics. These should include populations expected to have undergone minimal anthropogenic impacts, mainland populations, the population on King Island with low or no MHC class II DZB gene diversity, populations known to be affected by the disease mucormycosis and populations facing specific threats from human land-use practices.

Changes in population age/sex structure, distribution, disease prevalence and genetic diversity have all been associated with declines in wildlife populations. Consistent with the concept of extinction debt (Ford et al., 2009; Hylander and Ehrlén, 2013) - demographics, disease prevalence and genetic diversity may be more sensitive to early conservation impacts than estimates of population size alone, particularly in species whose numbers undergo considerable natural fluctuations, or in species for which population size estimates are not reliable Most conservation research projects have focussed on one or two of these areas. However, without investigations into all of these areas, understanding of the processes involved with population declines may be less complete than it could be. Equally, without baseline data from stable populations it can be difficult to interpret data from possibly impacted populations. As such, the population health assessment framework presented in this project offers an important

methodology for use in both impacted and apparently stable wildlife populations, and is a template for future conservation research not only in platypuses but also in other wildlife species.

APPENDICES.

Appendix A contains a map of fieldwork site locations and images of nets at 74 of these sites

Appendix B describes the anaesthetic protocol that was developed for use in relatively cold field conditions, including monitoring procedures and potential complications.

Appendix C is a paper that has been published in Conservation Physiology (Macgregor *et al.*, 2014) which reports a detailed investigation of the characteristics, triggers and mechanism of apnoea and bradycardia under isoflurane anaesthesia in platypuses.

Appendix D contains the survey of public sightings information sheet and questionnaire.

Appendix E is a methods paper that has been published in Pacific Conservation Biology (Macgregor *et al*., 2015) describing the novel use of in-stream microchip readers to monitor platypus movements.

Appendix F provides brief details of a preliminary study of platypus longevity and survivorship using the in-stream microchip readers at a location away from the main study area where platypuses had been microchipped up to 17 years previously.

Appendix G provides the derivation of the formula used to calculate tail fat volume from ultrasound measurements.

Appendix H illustrates the development of a reference curve for a seasonally varying haematology parameter.

APPENDIX A.

Fieldwork sites

Figure A.1. The locations of platypus (*Ornithorhynchus anatinus*) capture/release fieldwork sites (labelled by site number) in the Inglis Catchment, Tasmania.

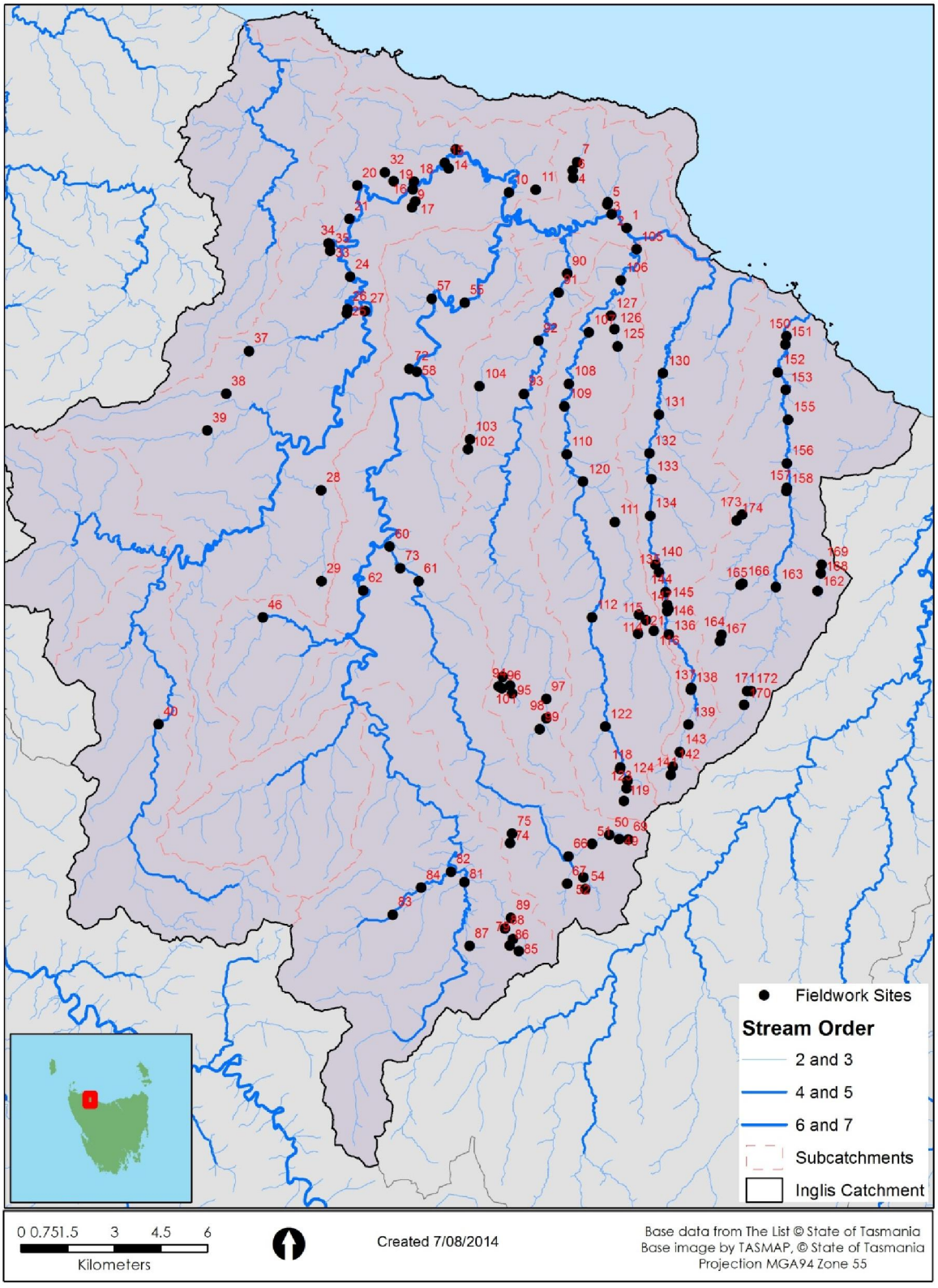


Figure A.2. Fieldwork sites.

Photo: Geoff Dutton

Figure A.2. (Cont'd) Fieldwork sites.

Photo: David McArtor

Photo: David McArtor

Figure A.2. (Cont'd) Fieldwork sites.

Photo: Deb Winfield

Figure A.2. (Cont'd) Fieldwork sites.

Photo: David McArtor

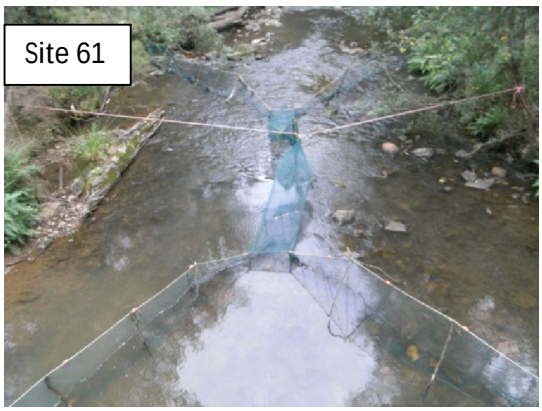


Figure A.2. (Cont'd) Fieldwork sites.

Photo: David Maleca

Photo: David Maleca

Figure A.2. (Cont'd) Fieldwork sites.

Figure A.2. (Cont'd) Fieldwork sites.

Photo: Sarah Munks

Figure A.2. (Cont'd) Fieldwork sites.

Photo: Christina Shaw

Photo: Sarah Munks

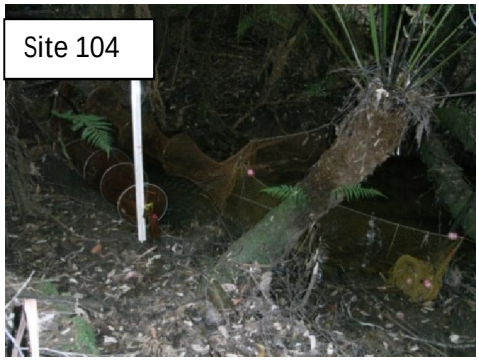


Figure A.2. (Cont'd) Fieldwork sites.

Figure A.2. (Cont'd) Fieldwork sites.

Photo: David Maleca

Figure A.2. (Cont'd) Fieldwork sites.

Photo: Geoff Dutton **Photo: Geoff Dutton**

Figure A.2. (Cont'd) Fieldwork sites.

Appendix B

Field anaesthesia of the platypus (*Ornithorhynchus anatinus*) in Tasmania, with particular reference to thermoregulation.

B.1 INTRODUCTION

Field anaesthesia of wildlife species is an important technique for allowing individual animals to be handled and examined, and samples taken or monitoring devices applied, with minimal pain or distress and to allow researchers to do so safely (Animal Care and Use Committee, 1998; Franson *et al.*, 1999; Fahlman *et al.*, 2008). In the platypus, general anaesthesia using halothane and ether by inhalation, and alfaxolone/alfadolone by intramuscular injection have historically been reported (Whittington and Grant, 1983; Grigg *et al.*, 1992; Evans *et al.*, 1994). The current method of choice for induction and maintenance of general anaesthesia in platypuses is isoflurane administered in oxygen via face mask (Booth, 2003; Booth and Connolly, 2008; McKee, undated), however its use in Tasmanian field conditions has not been reported.

The two main complications of isoflurane anaesthesia that have been reported are 1) hyperthermia, and 2) apnoea with marked bradycardia that has been referred to as inappropriate initiation of the dive response (Booth, 2003; Booth and Connolly, 2008; McKee, undated). Issues surrounding the occurrence of apnoea and marked bradycardia are discussed in Appendix C (Macgregor *et al.*, 2014). The aim of this aspect of the project was to develop a protocol for the safe field anaesthesia platypuses under Tasmanian conditions and to make observations based on anaesthetic monitoring records with particular reference to thermoregulation.

B.2 METHODS

A total of 166 platypus field anaesthetic procedures were performed in the Inglis River Catchment (41.06°S, 145.64°E) in Tasmania between August 2011 and August 2013 as part of a research project assessing the health of wild platypus populations. Platypuses

were examined in the field close to the site of their capture either in a tent erected on-site, or in a farm shed (if available). After capture in a fyke net (Macgregor *et al.*, 2010), each platypus was held in a cotton bag inside a hessian sack, in a parked vehicle, field examination tent or farm building so the animal was protected from adverse weather conditions, for a minimum of 60 mins (mean = 125 ± 50, max = 324, min = 60) to allow its fur to dry and its stomach to empty (Booth and Connolly, 2008) before being anaesthetised for examination (See Figures 2.5, 2.6 and 2.7 for images of platypus handling and anaesthetic techniques).

For all except the first procedure the following methods were used to assist thermoregulation before, during and after anaesthesia. When the air temperature was below 10°C, during holding periods before and after anaesthesia a 32-34°C hot water bottle was placed between the cotton bag and hessian sack in a position where the platypus could chose to be close to it, or move away from it. Body temperature was maintained under anaesthesia when necessary by a thermostatically-controlled heat pad and a bubble wrap blanket (See Figures 2.7 and C.1). For the first procedure, no external heat source was provided for the platypus during holding before anaesthesia despite the temperature being lower than 10°C. During anaesthesia of this platypus, maintenance of body temperature was attempted using fabric blanket and a hot water bottle in a cut-out section of a foam block on which the platypus was placed, with 2cm of foam between it and the platypus.

For all platypuses, anaesthesia was induced with 5% isoflurane in oxygen at 2 L/min by face mask, and was generally maintained with isoflurane at 1.5%, in oxygen at 1.5

L/min. Clinical judgement based on anaesthetic monitoring was used to guide the timing of changes in the delivered concentration of isoflurane from induction to maintenance and whether any changes were required during maintenance. The following parameters were assessed and recorded every 5 min: heart rate by auscultation, respiratory rate by visual examination, cloacal body temperature using a Welch Allyn SureTemp® Plus 690 Electronic Thermometer (Welch Allyn, Skaneateles Falls, USA) and anaesthetic depth by response to stimuli. Measurement of pulse rate and peripheral oxygen saturation was attempted early in the project using a Nellcor N-65 pulse oximeter (Covidien plc., Dublin, Ireland). A Parks model 811-b doppler ultrasound (Parks Medical Electronics Inc., Aloha, OR, USA) was used for continuous monitoring of pulse rate during the last ten anaesthetic procedures. Recovery from anaesthesia was achieved by stopping the delivery of isoflurane. Recovery from anaesthesia was determined to have occurred when the platypus' eyes opened. Recovery time was defined as the time in minutes from the end of isoflurane delivery until the platypus opened its eyes. Platypuses were held for one hour following recovery before release, each at the site of its capture (See Figure 2.10).

Platypus weight and age/sex category were determined as described in Section 2.2.5. The minimum temperature recorded at Wynyard (Climate data online, 2014) for each night of fieldwork was used as a measure of ambient temperature.

B.3 RESULTS

The age/sex category details and number of anaesthetic procedures for each individual were as follows: 61 adult females, three juvenile females, 68 adult males, six subadult males, six juvenile males each anaesthetised on one occasion, two adult females, six

adult males each anaesthetised on two occasions; two adult males each anaesthetised on three occasions. No platypus deaths or other long-term adverse consequences were recorded during the fieldwork. Details of the pre-anaesthetic holding times and anaesthetic duration are shown in Table B.1.

Table B.1. Details of pre-anaesthetic holding times, anaesthetic durations and ambient temperatures.

	Mean	SD	Max	Min
Holding time before anaesthesia (min)	124.7	49.5	324	60
Duration of isoflurane anaesthesia (min)	25.1	6.0	40	9
Nightly minimum temperature at Wynyard	7.3	5.2	19.9	-1.8

B.3.1 General observations

Several general observations were made during this aspect of the project that have been listed below to assist future researchers.

- The typical range for heart rate during maintenance of stable anaesthesia was 114-162 bpm. The typical range for respiratory rate during maintenance of stable anaesthesia was 6-24 breaths per minute.
- During the course of a standard anaesthetic procedure, the maintenance isoflurane concentration that was generally used was 1.5%. Occasionally platypuses moved slightly during examination, or showed gradual changes in heart and/or respiratory rate but only rarely was a change in maintenance isoflurane concentration from 1.5% required to allow the examinations/procedures detailed in Section 2.2.5 to be performed.
- Cloacal temperature fell relatively rapidly during anaesthesia if external heating and a bubble wrap blanket were not used (See Section B.3.3 for details of three platypuses that were exceptions to this generalisation).

- A heat pad setting of 32-34°C and a bubble wrap blanket was adequate to elevate cloacal temperatures of below 30°C or maintain cloacal temperatures of 30-34°C while the platypus was in sternal recumbency.
- Platypuses lost heat more quickly when in dorsal recumbency than in sternal recumbency. When in dorsal recumbency, cloacal temperature frequently fell despite the use of a heat pad setting of 34°C and a bubble wrap blanket.
- Pulse oximetry was rarely effective with the probe placed on the foot webbing, the dorsal bill shield or ventral bill shield. It was more likely to be effective when used on an unpigmented area (Figure B.1).
- Doppler ultrasound was very effective for measuring pulse rate. The probe was placed in the midline on the ventral surface of the tail (Figure B.2) and secured with tape. The positioning of the cloacal thermometer directly ventral to this ensured good contact of the probe to the skin.

Figure B.1 Unpigmented skin on the platypus (*Ornithorhynchus anatinus*) a) on the ventral aspect of the bill, and b) on the webbing of a forefoot.

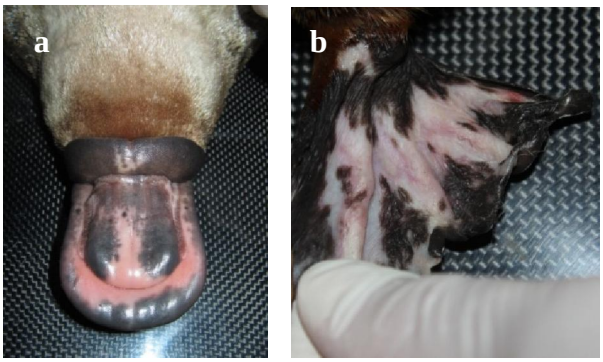
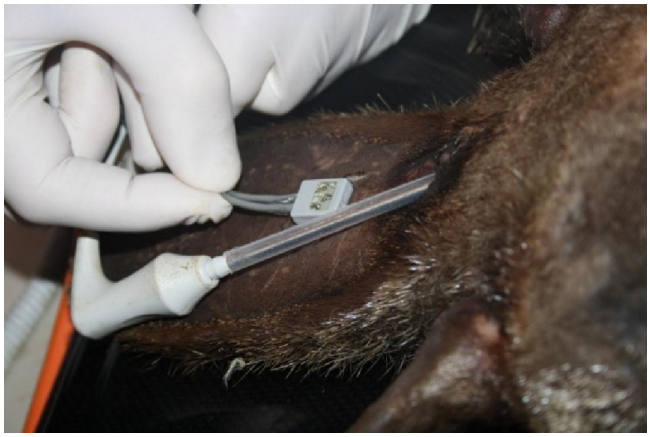


Figure B.2 Position of doppler ultrasound probe in the midline on the ventral aspect of the tail. **Photo: Geoff Duton.**



B.3.2 Platypus 1

The first anaesthetic procedure was performed on platypus 1, an adult female. Her body mass of 1.44kg was higher than the mean body mass of 1.32kg for female platypuses in this project. This platypus was removed from the net at 10pm. Anaesthesia was induced at 12 midnight, at which point the ambient temperature was measured to be 1°C, in a three sided tent erected ~500m from the site of her capture. For induction, 5% isofluorane was delivered in oxygen at 1.5L/min for 3 minutes. Anaesthesia was then maintained with 3% isoflurane for 12 minutes, as recommended by McKee (undated) to reduce the chance of apnoea and bradycardia, before being reduced to 2% for 15 minutes then being turned off for recovery. For all of this time the platypus was in sternal recumbency. During maintenance of anaesthesia her heart rate was steady at 120-150bpm. Application of the pulse oximeter probe to the foot webbing or bill shield did not produce any readings. The cloacal temperature shortly after induction was 27.7°C, and this dropped slowly to 27.2°C during the anaesthetic procedure.

Recovery from anaesthesia took approximately 120mins. During the first 90mins of recovery the heart rate (which was monitored almost constantly) was mostly in the range 60-100bpm, but

approximately every five minutes apnoea and more severe bradycardia occurred, sometimes with periods of 10-15s between heart beats. For the first 30mins of recovery, the platypus was held in the holding sacks on a hot water bottle placed between the sacks. Subsequently she was also taken into a stationary car (with the engine off) for additional protection from the cold. During the last 30mins, the periods of apnoea and bradycardia stopped and recovery from anaesthesia accelerated. This platypus was released at the site of her capture 60mins after full recovery from anaesthesia. She walked to the creek then swam away normally. The remote monitoring undertaken associated with this project demonstrated that 20 months after her capture, examination and release, she continued to regularly move past her capture site (Figure 3.6).

B.3.3 Body temperature

Figures B.3 and B.4, respectively, illustrate the first cloacal body temperatures of anaesthetised platypuses taken shortly after induction, and the last cloacal body temperatures taken shortly before the start of recovery.

Figure B.3. First cloacal body temperatures of anaesthetised platypuses in the Inglis catchment, Tasmania, taken shortly after induction.

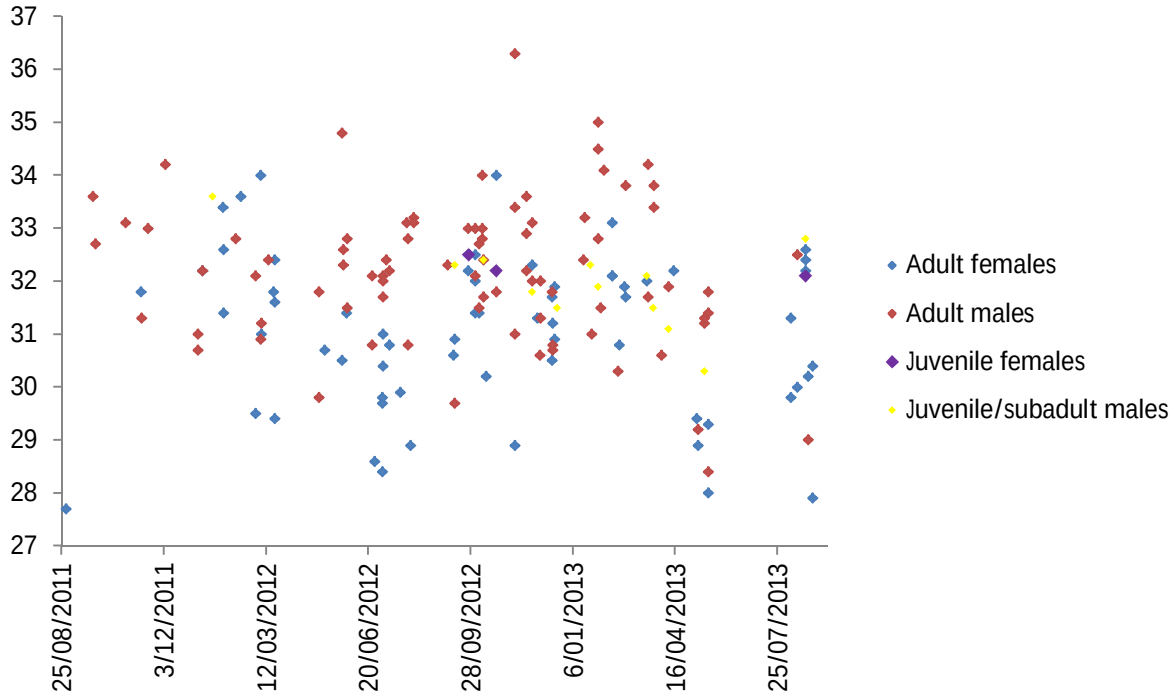
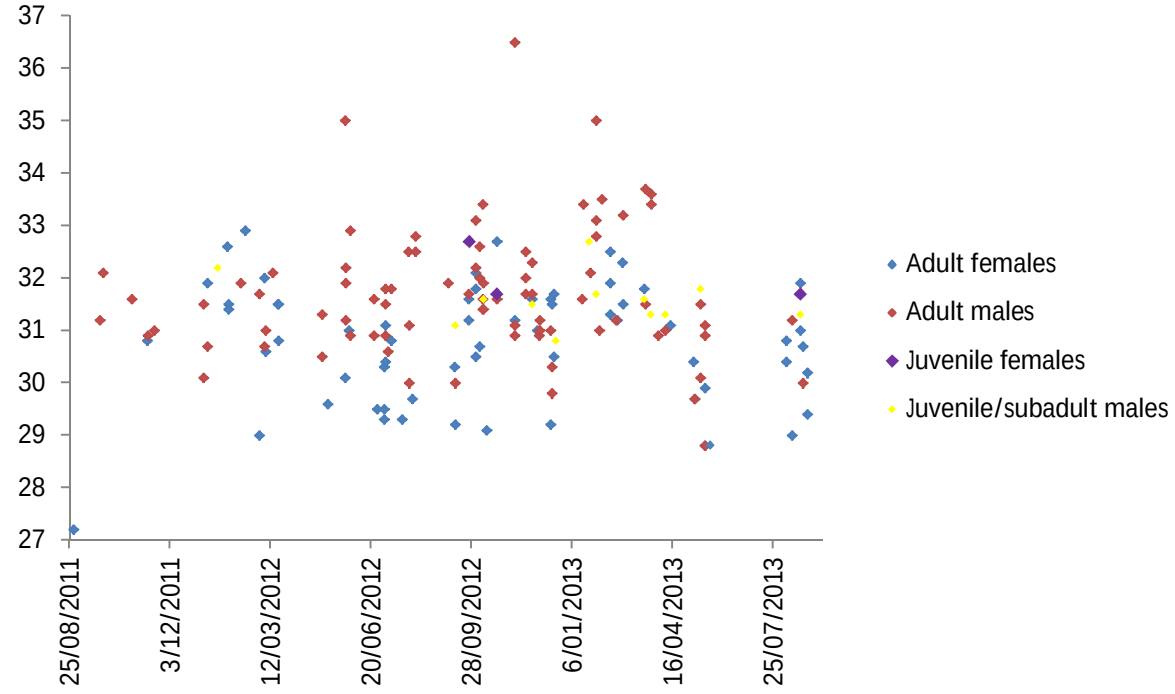


Figure B.4. Last cloacal body temperatures of anaesthetised platypuses taken shortly before the start of recovery.



The effects of the different protocol for Platypus 1 compared to all the other platypuses can be seen; her body temperature shortly after induction was the lowest encountered and, as described in Section B.3.2, this continued to fall during her anaesthetic procedure. The body temperature of the three platypuses (all adult males, subsequently referred to as hyperthermic) with the highest values shortly after induction that did not fall during anaesthesia, despite the heat pad being turned off and the bubble wrap blanket being removed, can also be seen.

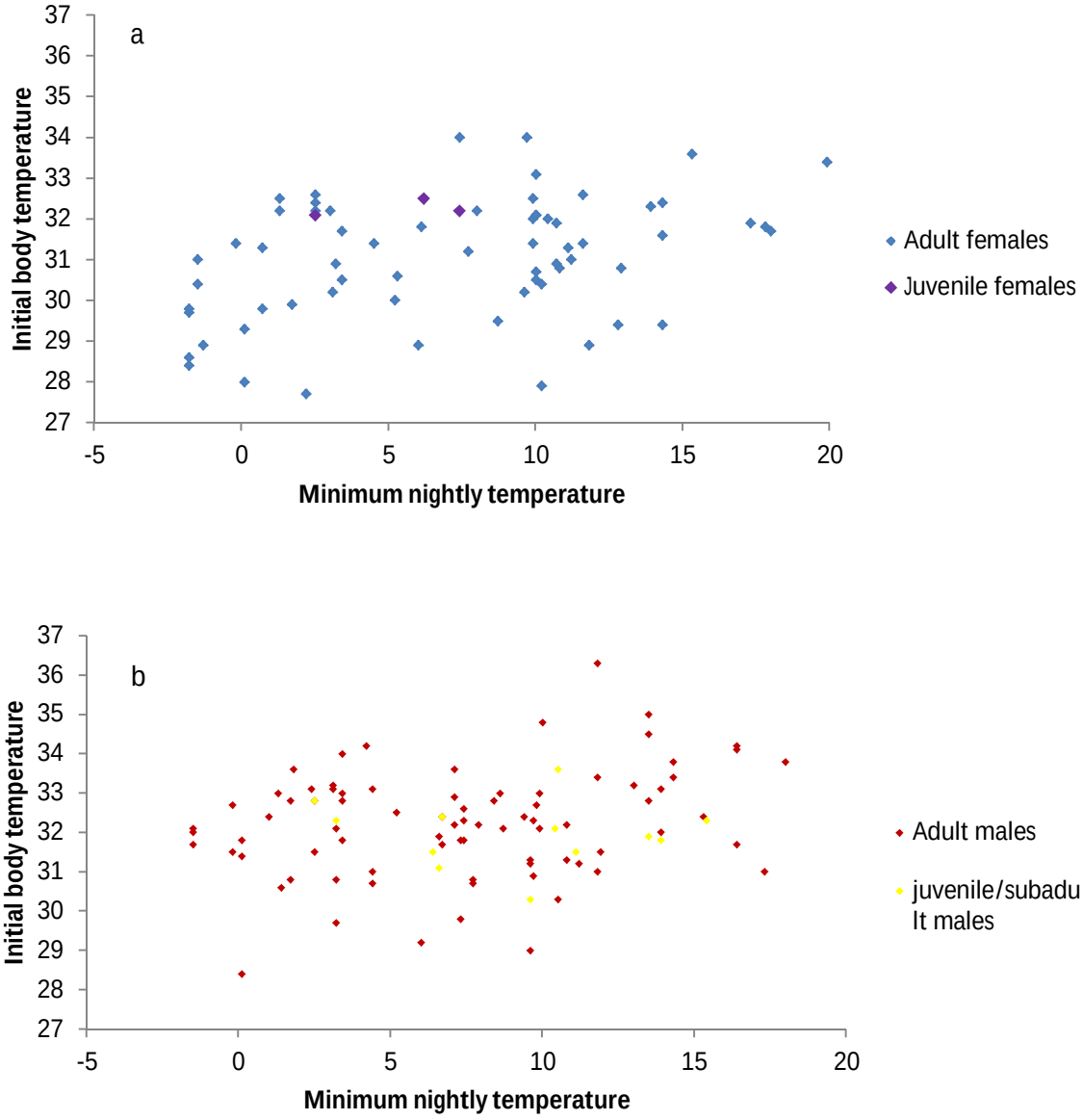
Excluding these four platypuses, the initial body temperatures ranged from 27.9°C to 34.5°C and showed a seasonal pattern with values tending to be higher in summer and lower during winter. Multiple regression with initial body temperature as the dependent factor and minimum nightly temperature, body mass, sex (male = 1, female =0) and age category (adult = 1, non-adult = 0) as independent factors demonstrated a significant effect of minimum nightly temperature and body mass and a near significant effect of age category, as shown in Table B.2.

Table B.2. Results of forward stepwise multiple regression with initial body temperature as the dependent factor and minimum nightly temperature, body mass, age category (adult = 1, non-adult = 0) and sex (male = 1, female =0) as independent factors.

N=157	Beta	Std.Err. of Beta	B	Std.Err. of B	t(153)	p-level
Intercept			29.720	0.465	63.904	<**0.001**
Body mass	0.412	0.072	1.191	0.208	5.725	<**0.001**
Min nightly temp	0.263	0.071	0.069	0.019	3.701	<**0.001**
Age category	-0.141	0.072	-0.658	0.335	-1.960	0.052

Figure B.5 shows the initial body temperature plotted against minimum nightly temperature for each sex.

Figure B.5 Initial body temperature plotted against minimum nightly temperature for a) males, and b) females.



In females, 15 platypuses (23%) had an initial body temperature of <30°C; the highest ambient temperature at which this occurred was 14.3°C. No females had an initial body temperature of >34°C. In males, five platypuses (6%) had an initial body temperature of <30°C; the highest ambient temperature at which this occurred was 9.6°C. A total of

seven male platypuses (9%) had an initial body temperature of >34°C. The lowest minimum nightly temperature recorded for three hyperthermic platypuses was 10°C. The four other males with initial body temperatures of >34°C were examined on nights with minimum temperatures of 4.2°C, 13.5°C, 16.4°C and 16.4°C.

Figure B.4 shows that, with the exception of Platypus 1 and the three hyperthermic platypuses, body temperatures shortly before recovery ranged from 28.8°C to 33.7°C. This range was narrower than that shortly after induction and demonstrates that the body temperature values at the upper and lower end of the range shortly after induction had been brought into line with the values of the other platypuses shortly before recovery.

B.3.4 Recovery time

The recovery times of those platypuses that did not undergo a nasopharyngeal response at recovery are shown in Figure B.6.

Figure B.6 Recovery time plotted against final body temperature for all platypuses that did not undergo a nasopharyngeal response.

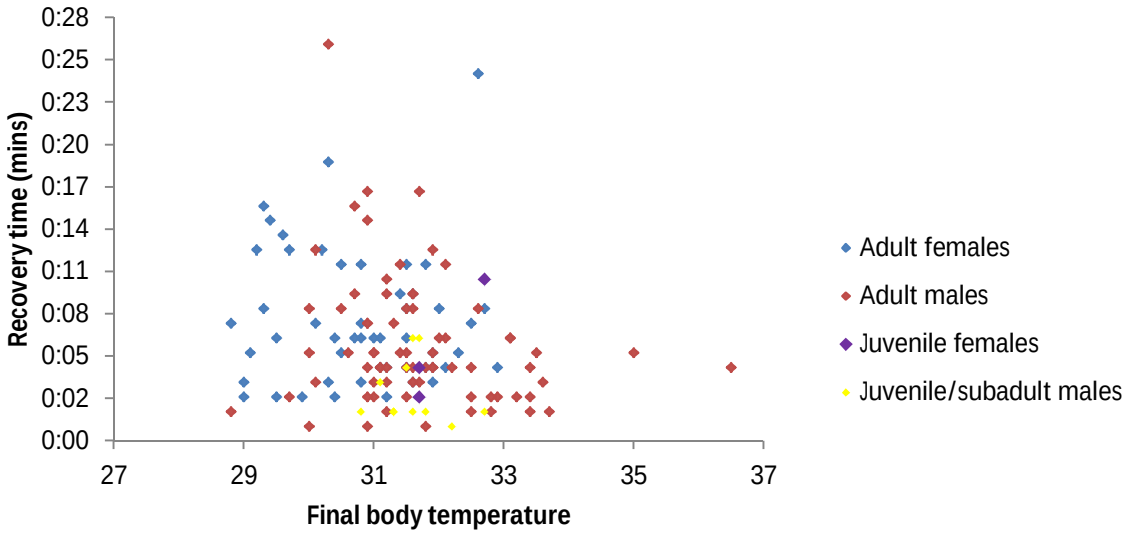


Table B.3 shows that forward stepwise multiple regression with recovery time as the dependent factor and final body temperature, duration of isoflurane delivery, age category (adult = 1, non-adult = 0) and sex (male = 1, female =0) as independent factors (with Platypus 1, the three hyperthermic platypuses, two platypuses with recovery times ≥25mins and platypuses that underwent a nasopharyngeal response excluded from the analysis) revealed a significant effect of both age category and final body temperature.

Table B.3 Results of forward stepwise multiple regression with recovery time as the dependent factor and final body temperature, duration of isoflurane delivery, age category (adult = 1, non-adult = 0) and sex (male = 1, female =0) as independent factors.

n=133	Beta	Std.Err. of Beta	B	Std.Err. of B	t(153)	p-level
Intercept			0.020	0.007	2.795	**0.006**
Age category	0.216	0.083	0.002	0.001	2.618	**0.010**
Final body temp	-0.207	0.089	-0.001	0.000	-2.312	**0.022**
Isoflurane delivery time	-0.142	0.084	0.000	0.000	-1.683	0.095
Sex	-0.121	0.088	-0.001	0.000	-1.374	0.172

B.4 DISCUSSION

This project has shown that the protocol used for all but the first anaesthetic procedure is a safe and effective method of anaesthetising platypuses in the field in the relatively cold Tasmanian climate. Analysis of the results provides insights into the issue of platypus thermoregulation during fieldwork involving live capture and release.

Grigg *et al.* (1992) found 98.5% of temperature recordings from freely ranging platypuses in winter in the Thredbo River to be between 30°C and 34°C. In this study, at the start of anaesthesia 23% of females had a temperature below this range, 6% of males had a body temperature below this range, and 9% of males had a body temperature above this range, despite efforts to prevent this occurring. Grant and

Dawson (1978a) found that the mean cloacal temperature of platypuses that had been entangled in nets for up to ~30mins in winter (30.4 ± 0.7°C) and summer (32.1 ± 0.7°C) were significantly different ($p<0.01$) They concluded that the loss of body temperature in their study likely resulted from disturbance of the insulating fur layer while platypuses were entangled and struggling in gill nets (Grant and Dawson, 1978a). This study found that the body temperature shortly after induction of anaesthesia was significantly correlated, positively with a measure of ambient temperature and positively to body mass, and this is consistent with a greater rate of heat loss at lower temperatures and in individuals with a greater surface area to volume ratio. In fyke nets, as used in this project, platypuses do not become entangled and fur disturbance is less likely to have occurred. This fieldwork was undertaken in the platypuses' natural environment and so ambient air/water temperatures during this fieldwork would be the same as those that the platypuses naturally encounter. However, it is thought that platypuses maintain their body temperature while swimming in cold water in part due to heat produced by muscle activity, and their burrow temperatures have been shown to maintain a relatively constant temperature of ~14-18°C which would reduce heat loss in winter (Grant and Dawson, 1978a; Grant and Dawson, 1978b; Bethge *et al.*, 2004). The absence during holding of platypuses in research projects of both heat from muscle activity and a constant warm burrow temperature, seems likely to predispose held platypuses to heat loss and, in conjunction with the observations of body temperature in this study, has implications both for studies using anaesthesia during platypus examination and for those examining conscious animals. Figure B.5 suggests that 10°C is approximately the environmental temperature below which male platypuses should be provided with an external heat source during holding, but 15°C may be more appropriate for females. Since hot water bottles between the holding sacks will

gradually cool down during holding, a thermostatically controlled heat source of a suitable size and to which power can feasibly be provided in the field may be more appropriate. For males there was overlap between the nightly minimum temperature range when some individuals had an initial body temperature of <30°C and when some individuals had an initial body temperature of >34°C. As a result, providing an external heat source to males, and possibly to females, may increase the chances of platypuses having a body temperature of >34°C during holding and care should be taken in doing so.

The fact that the three platypuses referred to as hyperthermic in this study not only had the three highest temperatures at observed induction, but also did not lose heat in the absence of an external heat source or thermal insulation suggests that the observed body temperatures in these individuals may reflect a phenomenon different to a simple inability to lose heat at warmer ambient temperatures. Psychological stress leads to hyperthermia in a range of mammalian species (Groenink *et al.*, 1995). Platypuses are known to be very susceptible to stress during capture, holding and handling (Whittington and Grant, 1995; Munday *et al.*, 1998), and this was a likely cause of the persistent hyperthermia in these three individuals. Hyperthermia has been reported as one of the two main potential complications of platypus anaesthesia (Booth and Connolly, 2008; McKee, undated). However, temperatures up to 35.5°C have also been reported in platypuses during holding after capture (Grigg *et al.*, 1992). While reduced thermoregulatory control during anaesthesia (Buggy and Crossley, 2000) may make hyperthermia a particular concern during anaesthesia, it seems likely that stress during capture/holding is an important initiating factor, and the observations from these three

platypuses suggest that hyperthermia is an additional reason to avoid stress in all captured platypuses.

Isoflurane and other anaesthetic agents have been reported to lead to peripheral vasodilation, which in combination with exposure to cool environments often lead to perioperative heat loss in humans (Xiong *et al.*, 1996; Sessler, 1997). The relatively rapid falls in body temperature that were observed under anaesthesia in the absence of an external heat source and/or a bubble wrap blanket in all but the three hyperthermic platypuses are consistent with this feature of anaesthetic agents, including isoflurane, as well as the low ambient temperatures and the small size of the platypus. Grant and Dawson (1978b) found the insulation provided by the fur on the ventral body fur to be greater than that of the dorsal body fur. They found little evidence of an insulative layer of fat and concluded that body fur was the most important method of insulation (Grant and Dawson, 1978b). They also found that at temperatures of 5-15°C the temperature of the skin on the ventral surface of the tail, which is usually unfurred, was maintained at close to ambient temperature but that at higher temperatures the temperature of the skin of the ventral surface of the tail could be elevated above ambient temperatures so that it is closer to the core body temperature (Grant and Dawson, 1978b). It was postulated that this may be achieved as part of normal thermoregulation by diverting blood via or around the rete mirabile countercurrent system in the caudal platypus (Grant and Dawson, 1978b). During anaesthetic procedures for this project, the ventral surfaces of the platypuses' tails were in contact with the heat pad when they were in sternal recumbency and were exposed to the air or loosely covered by a bubble wrap blanket when in dorsal recumbency. Given the higher insulative qualities of the ventral fur compared to the dorsal fur, and the effects of isoflurane on peripheral blood vessels, it

seems likely that alterations in the control of blood flow to and from the tail may have led to increased skin temperatures on the ventral aspect of the tail which, in turn, lead to increased heat loss when platypuses were in dorsal recumbency.

Hypothermia is known to prolong the duration of recovery from the effects of isoflurane and a wide range of other anaesthetic agents, for reasons including increased potency and decreased metabolism of anaesthetic agents, as well as increased inhalation agent blood solubility with consequent slower elimination via the lungs (Vitez *et al.*, 1974; Lenhardt *et al.*, 1997; Lockwood *et al.*, 1997; Pottie *et al.*, 2007) The significant correlation of recovery time with final cloacal temperature is consistent with this aspect of isoflurane pharmacokinetics. The extremely prolonged recovery with intermittent apnoea and varying degrees of bradycardia observed in Platypus 1 are consistent with a combination of the effects both of hypothermia on isoflurane pharmacokinetics and the nasopharyngeal response as a result of exposure of the nasal cavities to isoflurane and cold temperatures (See Appendix C). In other platypuses, nasopharyngeal responses during recovery only occurred in platypuses that had been placed in dorsal recumbency for ultrasonography. This may have been influenced by hypoxia and hypercapnoea occurring as a result of either poor upper airway patency or ventilation-perfusion ($\dot{V}/\dot{Q}$) mismatch in the lungs (See Section C.5). Platypus 1 had not been placed in dorsal recumbency during her examination. However, her body temperature was at least 1.6°C less than that of final body temperatures of all the other body temperatures. The observation of a nasopharyngeal response in this platypus is consistent with the suggestion that temperature influences the occurrence of this response (See Section C.5).

Most research projects undertaken in recent years have examined platypuses without the use of anaesthesia despite the well reported susceptibility of the species to stress in these situations. During examinations involving minimal procedures such as morphometrics and microchip insertion, it is likely that little additional stress reduction would be provided by anaesthesia. However, for projects undertaking more involved procedures (such as blood sampling, tissue biopsy, injections and dorsal recumbency) two reasons for the recent lack of the use of anaesthesia seem likely 1) inexperience of researchers with performing anaesthesia, and 2) concerns about anaesthetic risk. The former is not easy to overcome. In relation to the latter, while anaesthetic risk always exists, the demonstrated effects of stress on platypuses (Whittington and Grant, 1995; Munday *et al.*, 1998), which is likely to occur during examination and sampling, should not be ignored. No platypus deaths or other ongoing adverse effects occurred during the fieldwork and this section of the project has demonstrated that it is possible to safely anaesthetise platypuses for examination. Analysis of anaesthetic monitoring data has demonstrated the importance, for research projects that examine platypuses both with and without anaesthesia, of reducing stress in captured platypuses and carefully providing heat to platypuses in cold field conditions.

Appendix C

Investigation into the characteristics, triggers and mechanism of apnoea and bradycardia in the anaesthetised platypus (*Ornithorhynchus anatinus*).

This Appendix has been published as an original research paper in Conservation Physiology. Citation:

Macgregor JW, Holyoake C, Fleming PA, Robertson ID, Connolly JH, Warren KS (2014) Investigation into the characteristics, triggers and mechanism of apnoea and bradycardia in the anaesthetized platypus (*Ornithorhynchus anatinus*) *Conservation Physiology* 2: cou053

C.1 ABSTRACT

Health and conservation research of platypuses (*Ornithorhynchus anatinus*) may require anaesthesia to reduce stress and the risk of injury to both the animal and researcher, as well as facilitate examination and sample collection. Platypus anaesthesia can be difficult to manage, with reports of periods of apnoea and bradycardia described. This study investigated the conditions around sudden-onset apnoea and bradycardia in 163 field-anaesthetised platypuses as part of a health study. Anaesthesia was induced and maintained using isoflurane delivered in oxygen by face mask. Sudden-onset apnoea and bradycardia was observed in 19% of platypuses, occurring at either induction of anaesthesia, during recovery, or both. At induction, occurrence was more often recorded for adults ($p=0.19$), and was correlated with low body temperature ($p< 0.001$), season ($p=0.06$; greater incidence in summer), and longer pre-anaesthetic holding time ($p=0.16$). At recovery, sudden onset apnoea and bradycardia only occurred in platypuses that had been placed in dorsal recumbency as part of their examination, and correlated with poor body condition ($p=0.002$), time in dorsal recumbency ($p=0.005$), adults ($p=0.06$), number of fieldworkers ($p=0.06$) and females ($p=0.11$). The sudden onset apnoea and bradycardia we observed is likely to result from the irritant nature of isoflurane (stimulating the trigeminal nerve by nasal chemoreceptors). We propose that this mechanism is analogous to that of submersion of the face/nasal cavity in cold water during a natural dive response, but that the term 'nasopharyngeal response' would more appropriately describe the changes observed under isoflurane anaesthesia. Although we did not record any long-term adverse effects on platypuses that had undergone this response, the nasopharyngeal response could complicate the diagnosis of anaesthetic dose dependent apnoea and bradycardia. Therefore, we suggest that these responses

during anaesthesia of platypuses might be avoided by minimising the stress around capture and handling, as well as reducing time in dorsal recumbency.

C.2 INTRODUCTION

Anaesthesia is an important field technique for platypus health and conservation research, facilitating sample collection and measurements while minimising stress for the animal and reducing risk of envenomation for the researcher (McKee, undated). Apnoea and rapid onset bradycardia, with heart rates as low as 12 beats per minute (bpm) during anaesthesia have been recorded in platypus (Booth and Connolly, 2008; McKee, undated). These changes have been reported to be self-resolving, but to complicate the diagnosis of dose dependent apnoea and bradycardia which continue to deteriorate unless the dose is reduced. Apnoea and profound bradycardia under anaesthesia have also been reported in marine mammals, and have been identified as the cause of anaesthetic deaths (McDonell, 1972; Gales and Burton, 1988; Phelan and Green, 1992). In both platypuses and marine mammals, these respiratory and cardiac changes under anaesthesia have been described as an inappropriate initiation of the dive response (Lynch *et al.*, 1999; McKee, undated).

The dive response (or 'dive reflex' in older literature and some human literature; Foster and Sheel, 2005; Panneton, 2013) is a group of physiological changes that occur during submersion in water and has been described in a wide variety of aquatic and terrestrial vertebrate species (Irving *et al.*, 1941; Foster and Sheel, 2005; Panneton, 2013). The changes are broadly similar between species and consist of apnoea, bradycardia and compensatory redistribution of blood to the heart and central nervous system (CNS) by

peripheral vasoconstriction (Zapol *et al.*, 1979; Kooyman *et al.*, 1981; Foster and Sheel, 2005; Panneton, 2013).

Studies in a variety of mammalian species have investigated diving and the associated cardiovascular and respiratory effects, including apnoea, hypoxia, hypercapnoea, contact with water, temperature change and increased external pressure on the body. While there are differences between species, some general observations have been made. Apnoea occurs due to a combination of conscious and reflex control (Butler and Jones, 1997; Foster and Sheel, 2005), where the reflex response is triggered by trigeminal nerve stimulation as a result of submersion of the face and/or nasal passages ('facial submersion') in water (particularly cold water) (de Burgh Daly *et al.*, 1977; Drummond and Jones, 1979). Apnoea is the strongest trigger of bradycardia at the start of a dive (Foster and Sheel, 2005), although facial submersion can lead to bradycardia even when respiration is allowed to continue (Drummond and Jones, 1979; Foster and Sheel, 2005), and facial submersion and apnoea are synergistic on the development of bradycardia (Foster and Sheel, 2005). In fact, Irving *et al.* (1935) considered the dive response to be a modification of the physiological compensation during asphyxia. Apnoea results in changes in blood gas levels and detection of these changes by the carotid bodies becomes increasingly important in maintaining bradycardia (de Burgh Daly *et al.*, 1977; Lin *et al.*, 1983). An additional effect of conscious awareness on bradycardia is suggested by two factors. Firstly, the degree of bradycardia during a dive response has been observed to increase when an animal is forcibly submerged or when an animal dives because it is exposed to a threatening stimulus (Butler and Jones, 1997). Secondly, anticipatory changes have been observed in some species, with bradycardia occurring or resolving shortly before diving or surfacing, respectively (Thompson and

Fedak, 1993; Butler and Jones, 1997; Panneton, 2013). While afferent control by trigeminal nerve and carotid body stimulation is understood (de Burgh Daly *et al.*, 1977; Foster and Sheel, 2005; Panneton, 2013), and the efferent parasympathetic control of bradycardia and sympathetic control of compensatory vascular changes are known (Foster and Sheel, 2005; Panneton, 2013), there is less understanding of the central neural networks involved (Panneton, 2013). Given the number of stimuli involved and their changing relative effects, these neural networks are likely to be complex (Foster and Sheel, 2005).

Cardiac, respiratory and vascular changes similar to those of the dive response have been described in other situations, as a result of stimulation of the trigeminal nerve by mechanical or chemical receptors, e.g. in response to upper airway irritants ('nasopharyngeal reflex'; Yu and Blessing, 1997), mechanical stimulation of ocular and periocular structures ('oculo-cardiac reflex'; Schaller, 2004), or direct stimulation of any part of the sensory trigeminal nerve pathway ('trigeminocardiac reflex'; Schaller *et al.*, 2009). Schaller (2004, p.663) considered these reflexes and the dive response to have "at least partially similar physiological mechanisms" and Gorini *et al.* (2009, p1443) described the dive response as "a subset of the trigeminocardiac reflex". However, in the absence of a comprehensive understanding of the mechanisms of any of these events, it is not possible to know whether they are identical, or just similar, responses to stimulation of the trigeminal nerve.

The platypus is found associated with inland water bodies in Eastern Australia and spends its time either feeding in water or resting in a burrow (Grant and Bishop, 1998).

Platypuses are generally out of their burrow each day for a single period of 7.3-16 h (Serena, 1994; Gust and Handasyde, 1995; Otley *et al.*, 2000; Bethge, 2002). A platypus must dive to the bottom of the water body to find and collect its food (Grant, 2007), returning to the surface to breathe and masticate the food. Kruuk (1993) observed an average dive time of 35 s, with a maximum of 75 s in wild platypuses. Evans *et al.* (1994) found that in captive platypuses most dives lasted between 30 s and 4 min, but observed a maximum dive time of 11 minutes. Bethge (2002) observed a mean dive depth of 1.21 metres and a maximum dive depth of 8.77 in wild platypuses.

Two studies have investigated the dive response in platypuses. Johansen *et al.* (1966) forcibly submerged two platypuses tied to boards. Heart rates were observed to slow gradually over 30-35s from pre-submersion levels of ~140bpm to minimum values of ~20bpm (Johansen *et al.*, 1966). Evans *et al.* (1994) used surgically implanted electrocardiogram/radio-transmitter systems to monitor the heart rate of five freely diving wild platypuses that had been brought into a captive situation. Pre-dive heart rates were in the range of 140-230bpm. Two patterns of heart rate changes were observed, associated with differing dive lengths. In dives of one to three and a half minutes, during which the platypuses were usually inactive, the heart rate decreased over ~30s and remained stable at 12-40bpm. During dives of up to one minute, heart rates were erratic but fell within seconds of diving to between 10 and 120bpm. Heart rates were generally lower during dives lasting 40-60s (20-80bpm) than during dives lasting up to 30s (70-120bpm) (Evans *et al.*, 1994).

McKee (undated) briefly described apnoea and bradycardia in anaesthetised platypuses. It was reported that these changes were likely to occur in individuals in a light plane of anaesthesia and usually resolved within three minutes without any reduction in anaesthetic dose. It was considered that an informal survey of four operators indicating that apnoea and bradycardia occurred in 1-2% of platypus inhalation anaesthetic procedures may have given an underestimate of the incidence due to the intermittent monitoring undertaken (McKee, undated).

The aim of this study was to examine the heart and respiratory rates of platypuses during isoflurane anaesthesia, currently the method of choice for platypus anaesthesia (Booth and Connolly, 2008; McKee, undated), to identify periods of sudden onset apnoea and bradycardia, to investigate factors by which they may be triggered and maintained, and to evaluate whether the term “dive response” is an appropriate description of these changes.

C.3 METHODS

A total of 163 platypus field anaesthetic procedures were performed in the Inglis River catchment (41.06°S, 145.64°E) in Tasmania between August 2011 and August 2013 as part of a research project assessing the health of wild platypus populations. Platypuses were examined in the field close to the site of their capture either in a tent erected on-site, or in a farm shed (if available). After capture in a fyke net (Macgregor *et al.*, 2010), each platypus was held in a cotton bag inside a hessian sack for a minimum of 1 h to allow its fur to dry and its stomach to empty (Booth and Connolly, 2008) before being anaesthetised for examination. When the air temperature was below 10°C, a 32-34°C

hot water bottle was placed between the cotton bag and hessian sack in a position where the platypus could chose to be close to it, or move away from it.

Anaesthesia was induced with 5% isoflurane in oxygen at 2L/min by face mask, and was generally maintained with isoflurane at 1.5%, in oxygen at 1.5L/min. Body temperature was maintained by a thermostatically-controlled heat pad and a bubble wrap blanket (Figure C.1), which were required due to the cooler environmental temperatures associated with anaesthesia of platypuses in the field in Tasmania. The following parameters were assessed and recorded every 5 min: heart rate by auscultation, respiratory rate by visual examination, cloacal body temperature using a Welch Allyn SureTemp® Plus 690 Electronic Thermometer (Welch Allyn, Skaneateles Falls, USA) and anaesthetic depth by response to stimuli. The same parameters were also checked at varying frequencies between scheduled recordings while health examinations were being performed. To avoid prejudging the nature of our observations, a new term, sudden-onset apnoeic/bradycardic event (SOABE), was used to describe an anaesthetic response involving apnoea (no breath over >1 min) and bradycardia (< 100 beats per minute, bpm) lasting >1min and occurring without any preceding or ongoing gradual decrease in heart rate and respiratory rate. Periods of apnoea and bradycardia lasting < 1 min were regarded as transient events which can be associated with anaesthesia in any animal. Observed gradual decreases in heart rate and/or respiratory rate were considered to be the result of anaesthetic dose dependent respiratory and cardiac depression and the isoflurane concentration was decreased. Heart and respiratory rates were monitored continuously or near continuously from the time an SOABE was suspected to the time eupnoea and eucardia were considered to have returned.

Figure C.1. Mask anaesthesia of a platypus (*Ornithorhynchus anatinus*) on a thermostatically-controlled heat pad with a bubble wrap blanket (not yet in place) to regulate body temperature, in the Inglis Catchment, Tasmania. **Photo: Christina Shaw.**

Independent-samples t-tests were conducted to compare the minimum heart rate during SOABEs at induction and during recovery from anaesthesia, and to compare the duration of SOABEs at induction and during recovery from anaesthesia (Statistica 8.0, Statsoft Inc., Tulsa, USA). The occurrence of SOABEs at induction and during recovery (yes or no) was compared separately with intrinsic and extrinsic factors using forward stepwise logistic regression (Statistica 8.0, Statsoft Inc., Tulsa, USA). For occurrence at induction, factors investigated were season of capture (summer or winter; September - February or March – August), number of fieldworkers (as a measure of the potential noise levels around capture and handling time), whether the platypus was alone in the net when it was found (yes or no), whether the platypus had been captured previously (yes or no), whether the platypus was transported in a car (yes or no; if not then it would

have been carried by hand in its holding sacks from the capture site to the examination site), number of platypuses captured in the fieldwork session, sex (male or female; spur present/absent) (Temple-Smith, 1973), the platypus's age (adult or non-adult; spur/spur sheath presence/morphology) (Temple-Smith, 1973 as modified by Grant, 1991), the platypus's body condition (1-5; tail volume index) (Grant and Carrick,1978), the platypus's initial body temperature during anaesthesia (°C), and holding time before anaesthesia (min). The relationships between SOABE occurrence at recovery tested the same factors, with the addition of duration of isoflurane administration (min) and estimated duration of dorsal recumbency for ultrasound examination (min) based on ultrasound procedures performed (0 = no procedures, 2 = tail fat ultrasound only, 6 = abdominal ultrasound only, 8 = both tail fat and abdominal ultrasound).

Values are expressed as means ± 1 standard deviation.

C.4 RESULTS

SOABEs were never recorded during maintenance of anaesthesia, but were observed in 31 anaesthetised platypuses (19% of the 163 tested), either during induction (n = 9 individuals) or recovery (n = 17 individuals) or both (n = 5 individuals). Because our monitoring was not continuous, the exact moment of transition from eupnoea to apnoea and from eucardia to bradycardia were not always observed. However, apnoea was observed to precede bradycardia on several occasions. On all other occasions apnoea and bradycardia had both already started at the time any changes were observed and it was not possible to determine whether or not one preceded the other. Transient apnoea and bradycardia was observed at induction or recovery in an additional 17 platypuses. There were no platypus deaths during the fieldwork.

Typical range for heart rate during maintenance of stable anaesthesia was 114-162bpm. During a SOABE sustained minimum heart rates were usually in the range 30-72bpm, but occasionally there was >10s between two consecutive heart beats. Typical range for respiratory rate during maintenance of stable anaesthesia was 6-24 breaths per minute. During a SOABE, respiration ceased for >1min. After a period of ~1-10min of apnoea and bradycardia, heart rate would gradually rise before regular breathing recommenced. A short period (~30-60s) of tachycardia and tachypnoea would then occur, following which the apnoea and bradycardia would frequently return, usually with a slightly higher heart rate than previously. This process of apnoea and bradycardia interspersed with tachycardia and tachypnoea would continue for up to 20min, with the periods of apnoea and bradycardia becoming steadily shorter and the bradycardia being less severe in successive periods, until eventually the platypus started breathing regularly and the bradycardia ceased. The lowest recorded heart rate during SOABEs was significantly lower at induction (42±21bpm) than for those recorded during recovery (61 ±18bpm; t_{33}=-2.85, p=0.007). There was no significant difference in the duration of SOABEs at induction (4±3min) or during recovery (11±7min; t_{10}=-1.77, p=0.107). During SOABEs at induction, platypuses generally retained some degree of voluntary movement and withdrawal reflex but when the SOABE ceased they quickly stabilised and became non-responsive under anaesthesia. At recovery, some platypuses that had shown initial signs of waking up became less alert and less responsive to stimulation after the onset of a SOABE. When the event ceased the platypuses awakened quickly.

Of the 163 anaesthetic procedures, 67 were performed on females (64 adult and 3 juvenile) and 96 were performed on males (84 adult, 12 juvenile/subadult). Other intrinsic and extrinsic factors of these platypuses are summarised in Tables C.1 and C.2.

Table C.1. Summary of categorical intrinsic and extrinsic factors relating to anaesthetised platypuses.

	Yes	No
Capture occurred in summer	85	78
Platypus alone in net when it was found	135	28
Platypus had been captured previously	12	151
Platypus transported in car	70	93

Table C.2. Summary of continuous intrinsic and extrinsic factors relating to anaesthetised platypuses.

	Mean	SD	Max	Min
Number of fieldworkers	3.1	1.0	6	2
Number of platypuses captured in the session	2.5	1.3	5	1
Body condition index (tail volume index)	3.2	0.9	5	1
Initial body temperature during anaesthesia (°C)	31.7	1.4	36.3	27.9
Holding time before anaesthesia (min)	124.7	49.5	324	60
Duration of isoflurane anaesthesia (min)	25.1	6.0	40	9
Duration of dorsal recumbency for ultrasound (min)	3.8	3.2	8	0

Table C.3. Summary of forward stepwise logistic regression for factors correlated with the occurrence of a SOABE at induction and during recovery. NA indicates factors that were not considered for the occurrence of a SOABE at induction; - indicate factors that did not improve the model during the stepwise analysis and were not included in the final model.

	Induction	Recovery
Season of capture (summer or winter)	p=0.06	-
Number of fieldworkers (count)	-	p=0.06
Platypus alone in the net when it was found? (yes or no)	-	-
Platypus had been captured previously (yes or no)	-	-
Platypus transported in a car (yes or no)	-	-
Number of platypuses captured in the fieldwork session (count)	-	-
Age (juvenile or adult)	p=0.19	p=0.06
Sex (male or female)	-	p=0.11
Body condition (tail volume index)	-	p=0.002
Initial body temperature during anaesthesia (°C)	p<0.001	-
Holding time before anaesthesia (min)	p=0.16	-
Duration of isoflurane administration (min)	NA	-
Duration of dorsal recumbency for ultrasound (min)	NA	p=0.005

The results from the forward stepwise regression (Table C.3) revealed that the occurrence of a SOABE at induction correlated with low body temperature ($p<0.001$). Although they did not yield statistically significant values, three other factors were also included in the final model; there was a greater occurrence in summer ($p=0.06$) and in

adults (p=0.19), and there was a positive correlation with increasing pre-anaesthetic holding time (p=0.16). Three of the four platypuses which had the lowest initial core body temperatures (<28.5°C) shortly after induction all had a SOABE at induction (Figure C.2). The remaining 11 platypuses with SOABEs at induction had body temperatures between 30°C and 34°C. No platypuses with initial body temperature >34°C had a SOABE at induction. SOABEs at recovery only occurred in platypuses that had been placed in dorsal recumbency as part of their examination. Occurrence correlated with time in dorsal recumbency (p=0.005) and poor body condition (p=0.002) in the forward stepwise regression model (Figures C.3 and C.4; Table C.3). Although they did not yield statistically significant values, three other factors were also included in this stepwise model; there was a positive correlation with the number of fieldworkers (p=0.06) and SOABEs were more frequent in adults (p=0.06) and in females (p=0.11).

Figure C.2. Occurrence of sudden-onset apnoeic/bradycardic event (SOABE) at induction of anaesthesia in 163 platypuses, shown by date and initial body temperature.

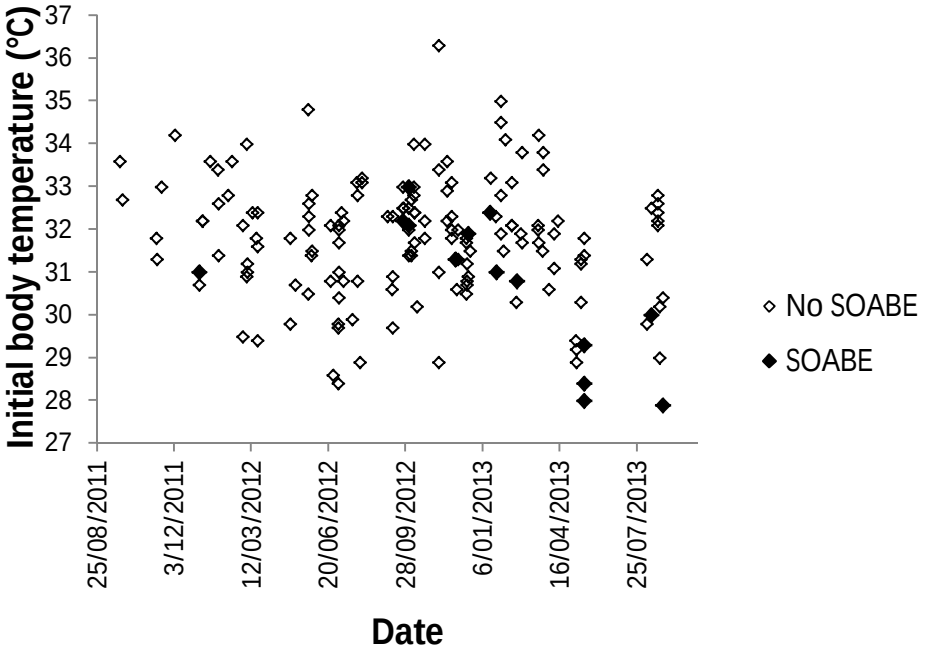


Figure C.3. Occurrence of sudden-onset apnoeic/bradycardic event (SOABE) at recovery from anaesthesia in 163 platypuses, shown by date and Tail Volume Index as a measure of body condition (5 = poorest body condition, 1 = best body condition).

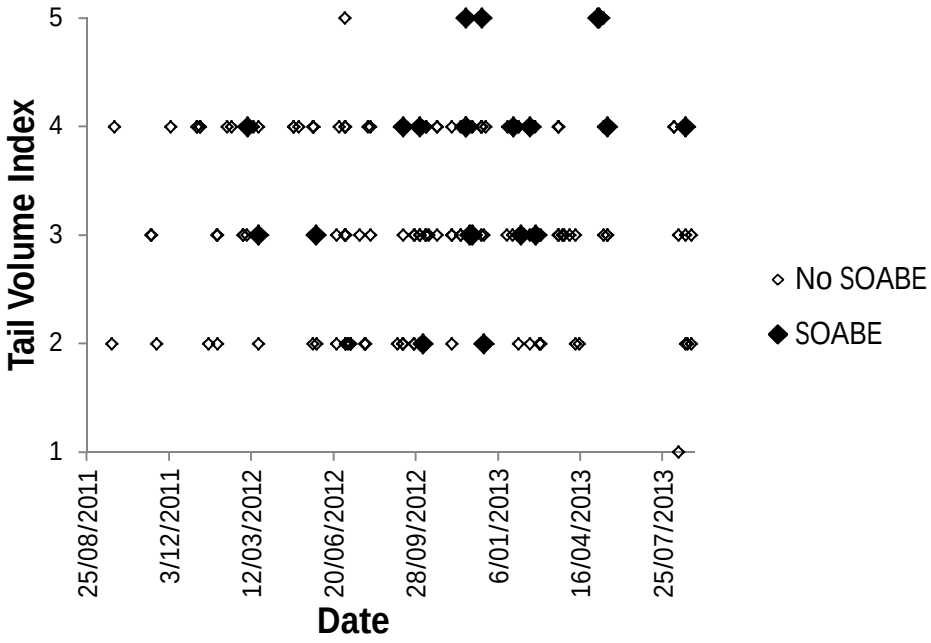
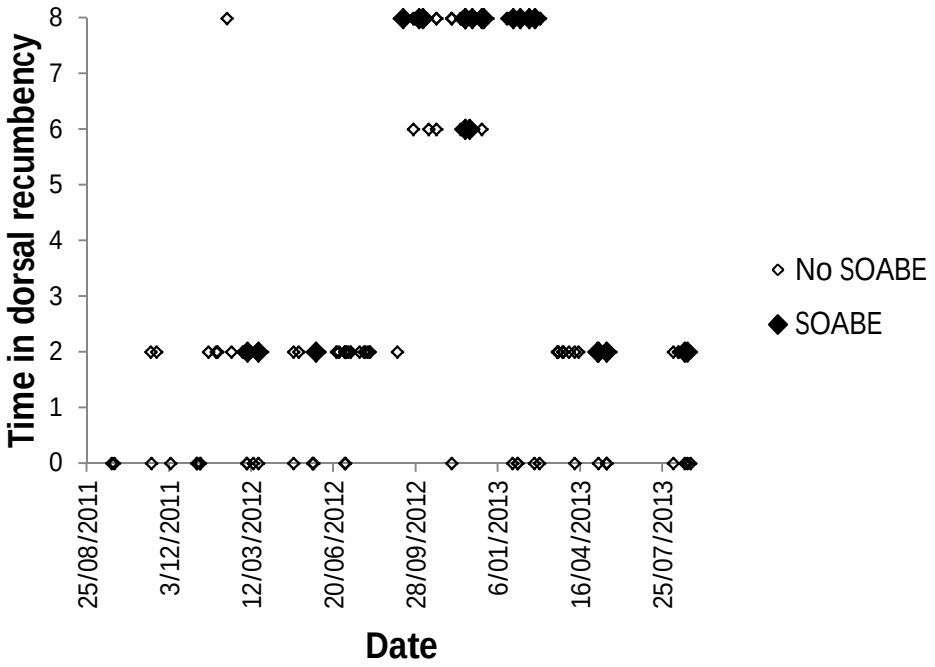


Figure C.4. Occurrence of sudden-onset apnoeic/bradycardic event (SOABE) at recovery from anaesthesia in 163 platypuses, shown by date and time in dorsal recumbency (see methods section for time estimation details).



C.5 DISCUSSION

In the following discussion we will show that the respiratory and cardiac characteristics of the SOABE we have described in platypuses during isoflurane anaesthesia are consistent not only with the dive response, but also with certain other previously described responses. We will propose that the mechanisms that trigger and maintain the SOABE during anaesthesia are similar to the control mechanisms that regulate the dive response but that there is a different primary trigger. Consequently we will suggest a change in nomenclature for this response. We will also discuss additional factors associated with incidence of SOABEs in platypuses, and discuss measures that can be taken during future studies to reduce their effects.

The SOABEs observed during anaesthetic induction or recovery in platypuses were consistent with those previously seen in voluntarily-diving platypuses, forcibly submerged platypuses, and naturally-diving seals (Table C.4) (Johansen *et al.*, 1966; Jones *et al.*, 1973; Evans *et al.*, 1994). They were also consistent with the changes recorded in rabbits during anaesthetic induction (Table C.5) (Flecknell *et al.*, 1996). Although blood pressure and blood gas analysis were beyond the scope of our project, the observations of these parameters by Flecknell *et al.* (1999) in rabbits were similar to those observed during diving in marine mammals. Flecknell *et al.* (1999, p.45) concluded that the mechanism for the apnoea, bradycardia, blood gas changes and blood pressure changes they observed during induction of isoflurane anaesthesia in rabbits appeared "to be similar to that associated with breathholding in man".

Table C.4. Characteristics of apnoea and bradycardia in voluntarily diving platypuses, forcibly submerged platypuses and naturally diving seals and platypuses during isoflurane anaesthesia.

	Voluntary dives of 40 – 60 s in platypuses in holding facility (Evans *et al.*, 1994)	Forcibly submerged platypuses (Johansen *et al.*, 1966)	Naturally diving harbour seals (Jones *et al.*, 1973)	Platypuses undergoing mask induction with isoflurane (This study)
Timing of bradycardia with relation to apnoea	Immediate onset, peaked after 15 s	Bradycardia started 5-10s after apnoea and peaked after 30-35 s.	Bradycardia started 2-3 s after submergence.	Sudden onset. Apnoea observed before bradycardia on occasion but never the reverse.
Minimum heart rate (% of initial heart rate)	~20	~12-37	~40-50	~30-45

Table C.5. Characteristics of the apnoea and bradycardia in rabbits and platypuses during isoflurane anaesthesia.

	Rabbits undergoing face mask delivered isoflurane anaesthesia (Flecknell *et al.*, 1996)	Platypuses undergoing face mask delivered isoflurane anaesthesia (This study)
Timing of apnoea/bradycardia	Induction	Induction and/or recovery
Onset of apnoea	Isoflurane concentration >0.5%	~4 min after start of induction or recovery
Minimum heart rate (% of resting heart rate)	18-45	At induction: ~30 At recovery: ~45
Duration of first period of apnoea and bradycardia (s)	30-120 s	60-600 s
Multiple periods of apnoea and bradycardia interspersed with short periods of tachypnoea and tachycardia	Yes	Yes
Level of consciousness during apnoea/bradycardia	Attempted to escape and pawed at nose.	At induction: eyes often open withdrawal reflexes intact, occasional spontaneous movements of head and legs. At recovery: often no movement but sometimes eyes open, withdrawal reflexes intact and/or arched back.
Proportion of animals affected	100%	9% at induction 13% at recovery

Isoflurane is known to cause airway irritation in a number of species and can lead to increased airway secretions as well as coughing and, most importantly in relation to the SOABEs we observed, breath-holding during light anaesthesia (Clarke and Trim, 2013). The degree to which this breath-holding is a conscious response is not known; however it has been shown that exposure of nasal receptors to airborne irritants in rabbits can, particularly in the conscious animal, trigger apnoea and bradycardia as a result of trigeminal nerve stimulation (Yu and Blessing, 1997). We hypothesise that in certain susceptible species, including the rabbit and platypus, stimulation of nasal receptors and the trigeminal nerve by isoflurane may not only lead to apnoea but also bradycardia, either as a secondary response to the apnoea or directly from stimulation of the nasal receptors (Figure C.5).

Although we believe that nasal irritation by isoflurane was necessary for development of the SOABEs we observed, our results indicate that the onset and maintenance of a SOABE was also influence by other factors. Similarly there appear to be factors other than facial submersion and apnoea that affect the onset and characteristics of the cardiovascular changes during diving. For example, Jones *et al.* (1973) observed that one harbour seal (*Phoca vitulina richardi*) showed no bradycardia in 20% of its feeding dives. Also, observed heart rates in forcibly submerged platypuses and inactive voluntarily diving platypuses in a holding facility have been be lower than those in active platypuses undertaking shorter voluntary dives (Johansen *et al.*, 1966; Evans *et al.*, 1994).

We acknowledge that in our study, not all SOABEs may have been recorded because our monitoring was not continuous and that our definition of a SOABE may have excluded some events that resulted from the action of isoflurane on nasal chemoreceptors. However, we are confident that no anaesthetic dose dependent apnoeic/bradycardic events were misclassified as SOABEs because at induction the bradycardia did not continue to deteriorate when respiration occurred despite maintained isoflurane levels, and at recovery the apnoea and bradycardia occurred after isoflurane delivery had ceased.

SOABEs were more likely to happen during induction if the initial body temperature of the platypus was low. This was heavily influenced by the fact that three of the four platypuses with lowest initial core body temperatures shortly after induction all had a SOABE at induction. The remaining 11 platypuses showing SOABE at induction had

body temperatures between 30°C and 34°C; Grigg *et al.* (1992) found 98.5% of temperature recordings from freely ranging platypuses in winter in the Thredbo River to be within the same limits. The occurrence of SOABEs in platypuses with apparently normal core body temperatures is consistent with the idea that low temperature was only one of a number of trigger factors for the SOABEs we observed. Alternatively it may be that core body temperature appeared to influence the rate of occurrence only because it was a proxy for the facial/nasal temperature, but that it was not a good one. Cold ambient temperature was a common feature of our fieldwork due to the geographical location. While we made attempts to maintain platypus body temperature to avoid hypothermia, we were cautious because hyperthermia is also a risk during platypus handling and anaesthesia (McKee, undated), particularly in captivity and warm environmental conditions.

SOABEs during recovery from anaesthesia only occurred in platypuses that had been placed in dorsal recumbency as part of their examination, and the rate of occurrence was related to the time each platypus spent on its back. Respiratory movements of the platypuses during dorsal recumbency appeared to be exaggerated and it may be that either poor upper airway patency or ventilation-perfusion ($\dot{V}/\dot{Q}$) mismatch in the lungs lead to increasing levels of hypoxia and hypercapnoea. Hypoxia and hypercapnoea have been observed to be involved in the development and maintenance of a natural dive response (de Burgh Daly *et al.*, 1977; Lin *et al.*, 1983); they may also have been important triggers for the apnoea and bradycardia that we observed. They may have been particularly important during recovery when the nasal isoflurane concentration would be expected to be lower than at induction, and when we had usually either partially or completely corrected any hypothermia present at induction. However, blood

gas changes would also have occurred at induction when apnoea occurred and if they were involved in developing and maintaining apnoea and bradycardia during recovery, they are likely to have had the same actions during induction. Other factors which may have resulted in the occurrence of a SOABE could be associated with an effect of increased stress levels in individuals at the time of anaesthesia in a similar manner to the effects observed in mammals and birds that dive naturally in response to threatening stimuli (Butler and Jones, 1997).

Two aspects of our results have no parallel during diving. The first of these is the cyclical nature of the apnoea and bradycardia. This was also seen during rabbit anaesthesia (Flecknell *et al.*, 1996) and provides strong evidence that the same phenomenon was being observed in the two species. It is likely that in the platypus the first period of tachycardia and tachypnoea occurs at roughly the time when the conscious animal would no longer be able to breath-hold during a dive and would come to the surface. The triggers for the break point of breath-holding are complex and not fully understood (Parkes, 2006). Prolongation of breath-holding times in humans with no apparent peripheral or central blood gas chemoreceptivity provide evidence that, in individuals with blood gas chemoreceptivity, hypoxia and hypercapnoea are involved with break point occurrence (Parkes, 2006). However, in general blood gas levels at break point are inconsistent between repeat breath-holds, or inspiration of varying levels of oxygen or carbon dioxide before breath-holding (Parkes, 2006). If blood gas pressures were the sole determinants of the break point timing it would not be possible to make a second breath-hold until these parameters had been improved. However, again in humans, Fowler (1954) demonstrated that allowing eight breaths of an asphyxiating gas mixture (8% oxygen, 7.5% carbon dioxide) at the break point of

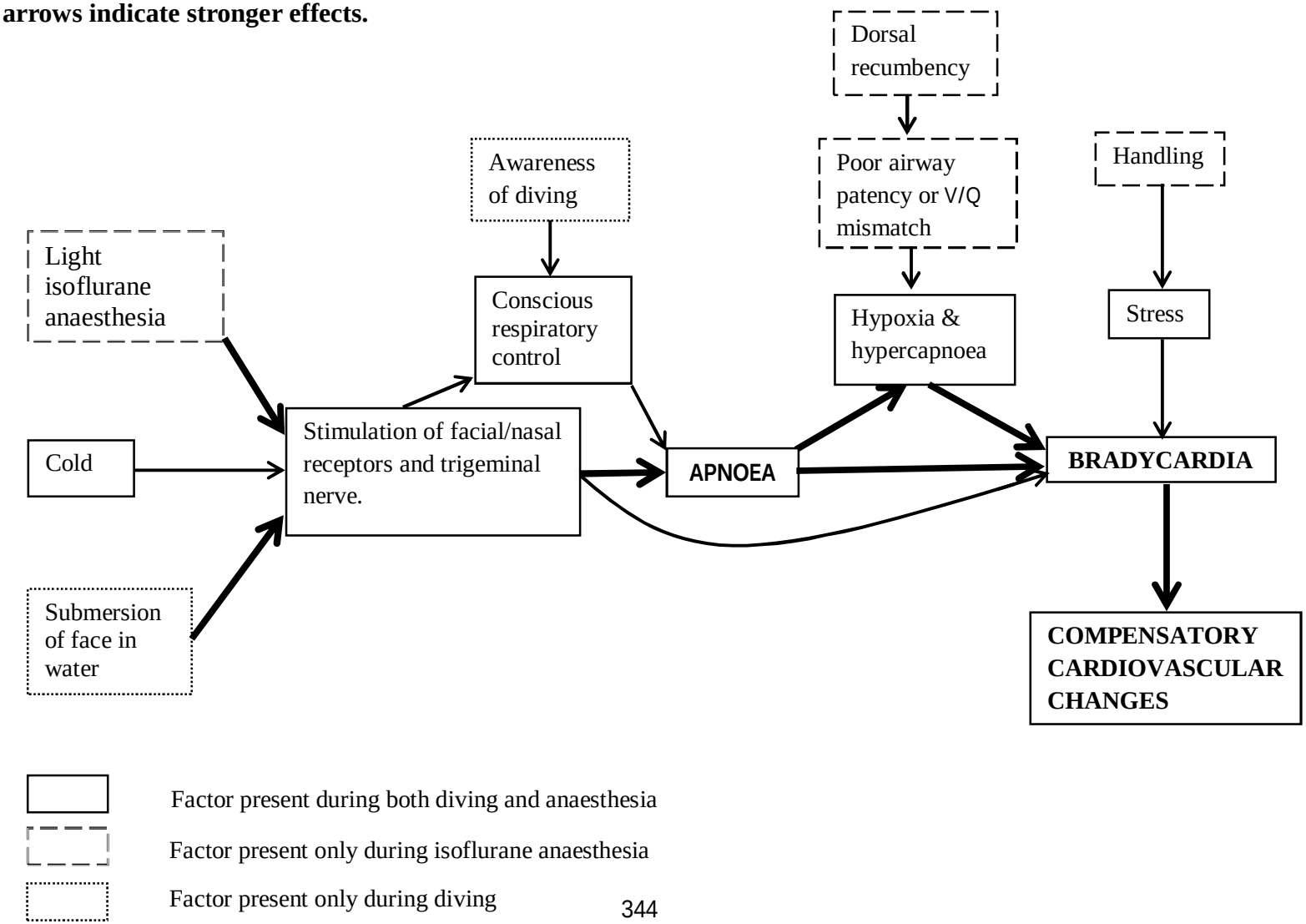
breath-holding allowed a second and then a third 20s breath hold to be performed. It has also been shown that a second breath-hold is possible if respiratory effort occurs against a closed airway and no gas is taken into the lungs (Rigg *et al.*, 1974). There may be a role diaphragmatic chemoreceptors and /or proprioreceptors as well as lung volume and metabolic rate in determining maximum breath-hold duration (Parkes, 2006). In particular, removal of diaphragmatic receptor stimulation may be the reason that a second breath-hold is possible despite ongoing hypoxia and hypercapnoea (Parkes, 2006). Regardless of the exact mechanism of breath-hold break point determination, it seems plausible that if the triggers that initiated apnoea and bradycardia in isoflurane anaesthetised platypuses remain after the stimuli to breath have been relieved, apnoea and bradycardia will recur.

The second observation that cannot be related to the naturally diving animal is that some platypuses became less alert and less responsive to stimulation after the onset of a SOABE during recovery. Since the trigeminal nerve mediated cardiovascular and respiratory responses are largely under parasympathetic control, this reduced responsiveness could have a similar mechanism to a freeze response which can occur as a response to fear, also under parasympathetic control (Hermans *et al.*, 2013).

We propose that, with the exception of transient anticipatory changes that have been described at the beginning and end of diving (Butler and Jones, 1997; Panneton, 2013), the overall mechanism of trigeminal nerve stimulation leading to apnoea and, either directly or secondarily to apnoea, to bradycardia (de Burgh Daly *et al.*, 1977), applies to the changes occurring in the platypus during natural diving and during light isoflurane

anaesthesia. This mechanism is shown in Figure C.5, which is in part a summary of the conclusions of previous research into the dive response. However, given that the primary trigger for trigeminal nerve stimulation under isoflurane anaesthesia is the presence of an irritant gas, and not the presence of water, in the nasal cavities we propose that the term ‘nasopharyngeal response’ would more appropriately describe the changes we observed.

Figure C.5. Path diagram for the development and maintenance of apnoea and bradycardia during diving and during isoflurane anaesthesia in the platypus. Bold arrows indicate stronger effects.



We consider it likely that the mechanism in Figure C.5 will also apply to other diving animals during isoflurane anaesthesia. However, given that we observed no platypus deaths and given the protective physiological nature of the responses to apnoea and trigeminal nerve stimulation discussed, the apnoea/bradycardia associated deaths reported by McDonell (1972), Gales and Burton (1988) and Phelan and Green (1992) would not be expected from such a response (Lynch *et al.*, 1999). Marine mammal anaesthesia faces a number of challenges including narrow anaesthetic agent dose ranges, a large amount of peripharyngeal tissue that can reduce airway patency, and the flexible nature of the thorax that requires considerable inspiratory effort to act against the weight of the thorax (Hammond and Elsner, 1977; Gage *et al.*, 2003; Tahmindjis *et al.*, 2003). It seems likely that the marine mammal deaths that have been reported to have been associated with apnoea and bradycardia have been at least in part associated with these other anaesthetic complications as reported by Tahmindjis *et al.* (2003). Apnoea and bradycardia have been reported in marine mammals as a consequence of injectable anaesthesia, during which nasal chemoreceptor stimulation would be absent. Seals can show periodic breathing with heart rate slowing during 20-40 s periods of apnoea (Thompson and Fedak, 1993), or can undergo physiological bradycardia during periods of apnoea of up to 20 min while resting on land (Andrews *et al.*, 1997), with similarities to the bradycardia observed during voluntary breath-holding and asphyxia in other species (Bauer, 1938; Bjurstrom and Schoene, 1987). Given the considerable ability of diving mammals to endure apnoea (Irving, 1939; Kooyman *et al.*, 1981) these species presumably have physiological mechanisms enhanced for this purpose and it would not be surprising if apnoea during marine mammal anaesthesia leads to bradycardia more readily than in terrestrial animals.

Further research is indicated to clarify the roles of stress, temperature and blood gases on the development of sudden onset apnoea and bradycardia during isoflurane anaesthesia in platypuses. However, a lower frequency of what we can now refer to as the nasopharyngeal response during platypus anaesthesia might be achieved by one or more of the following: use of a less irritant anaesthetic gas such as sevoflurane, premedication with injectable sedative(s), not placing platypuses in dorsal recumbency, further increasing efforts to mitigate stress on captured platypuses, and warming and humidifying inhaled gas.

Appendix D

Public survey information letter and questionnaire

School of Veterinary and Life Sciences
College of Veterinary Medicine
Murdoch University

Inglis River Catchment Platypus Survey

Thank you for taking time to look at this questionnaire. It has been prepared by James Macgregor as part of a study into the health of the platypus population in the Inglis River catchment and aims to investigate the distribution and abundance of platypuses in the area. Information gathered in this questionnaire will be related to the results of a field study being performed in the same area and also compared with the findings of similar questionnaire studies that have been performed elsewhere in Tasmania and mainland Australia.

The aim of the project is to gather information on waterbodies where you have seen one or more platypus as well as waterbodies where you have never seen a platypus. To gain some idea whether the information is current and historical, we have asked for information on the two time periods 1.1.2012 – 31.12.2012 and before 1.1.2012.

The questionnaire contains four tables that you can use to describe waterbodies that you have visited in the Inglis catchment (as shown on the map overleaf). Each of these tables can be used to describe either a site where you have seen a platypus or a site where you have never seen one. Please list any site where you have seen a platypus, regardless of how many times or how frequently you have visited it. Please only list sites where you have never seen a platypus if you consider yourself to be familiar with the location.

Along with the example (Site A) in the questionnaire, the following points may assist you to fill out the sections in the site tables.

1. Waterbody type eg. River, Creek, Dam – if the site is a dam, please indicate whether or not the dam is connected to a creek.
2. Waterbody name – if it has one.
3. Location of site – may describe a length of river or an area of a dam. Include a grid reference from Tasmanian 1:25,000 map if possible. Please also mark this location with a cross and a number on the map overleaf.
4. Please tick either “yes” or “no” for each time period.
5. Platypus sighting frequency – Please tick one box for each time period during which you have seen a platypus at this site. If you have not visited a site very often, you may not feel able to assess sighting frequency – in which case you can tick “Don’t know”.
6. Date and time of day of sightings. Please complete for each time period during which you have seen a platypus at this site. May be multiple dates or times if more than one sighting has occurred.

If you have more than four sites to list, or know anyone who else who might be able to provide information, more copies of the questionnaire can be obtained from Wynyard Veterinary Clinic, or by contacting James Macgregor on 0487 979 213 or platypusproject@hotmail.com . Alternatively, you could copy the questionnaire page.

Please return the completed questionnaire to James Macgregor at Wynyard Veterinary Clinic or PO Box 652, Wynyard, 7325 by 30th April 2013.

Platypuses are very secretive animals, and very little is known about their distribution and abundance at a catchment level. Thank you for any information you can give; it will be a great help to our understanding of this unique animal.

Please use this space to provide any further information that you feel may assist this research.
This may include sightings of platypuses on land, or dead on roads.

You can decide at any time to withdraw your consent to provide your platypus sightings information for our research. If you decide to withdraw, the information you have given us will be destroyed. My research supervisor Dr Kris Warren and I are happy to discuss with you any questions you may have about this study.

Sincerely

James W Macgregor

This study has been approved by the Murdoch University Human Research Ethics Committee (Approval 2012/222). If you have any reservation or complaint about the ethical conduct of this research, and wish to talk with an independent person, you may contact Murdoch University's Research Ethics Office (Tel. 08 9360 6677, or e-mail ethics@murdoch.edu.au). Any issues you raise will be treated in confidence and investigated fully, and you will be informed of the outcome.

Site A (Marked with a cross and an "A" on map overleaf) **[EXAMPLE]**

1. Waterbody type (eg River, Creek, Dam)	River
2. Waterbody name	Inglis River
3. Location of site	0-100 metres north of bridge at Bass Highway, west of Wynyard Grid ref (if known): 55390605(E) 5463104(N)
4. Have you seen a platypus at this site? (please tick)	1.1.2012 - 31.12.2012: þ Yes o No Before 1.1.2012 : þ Yes o No
5. If yes, what has been the frequency of these sightings? (please tick)	1.1.2012 - 31.12.2012: þ Rare o Common o Don't know Before 1.1.2012 : o Rare þ Common o Don't know
6. Approximate date and time of day of sightings (if known)	1.1.2012 - 31.12.2012: December 2011, early morning. Before 1.1.2012 : Most years since 1987. Usually early morning, but sometimes late afternoon or middle of the day.

Site 1 (Please mark with a cross and a "1" on the map overleaf)

1. Waterbody type (eg River, Creek, Dam)	
2. Waterbody name	
3. Location of site	Grid ref (if known):
4. Have you seen a platypus at this site? (please tick)	1.1.2012 - 31.12.2012: o Yes o No Before 1.1.2012 : o Yes o No
5. If yes, what has been the frequency of these sightings? (please tick)	1.1.2012 - 31.12.2012: o Rare o Common o Don't know Before 1.1.2012 : o Rare o Common o Don't know
6. Approximate date and time of day of sightings (if known)	1.1.2012 - 31.12.2012: Before 1.1.2012 :

Site 1 (Please mark with a cross and a "1" on the map overleaf)

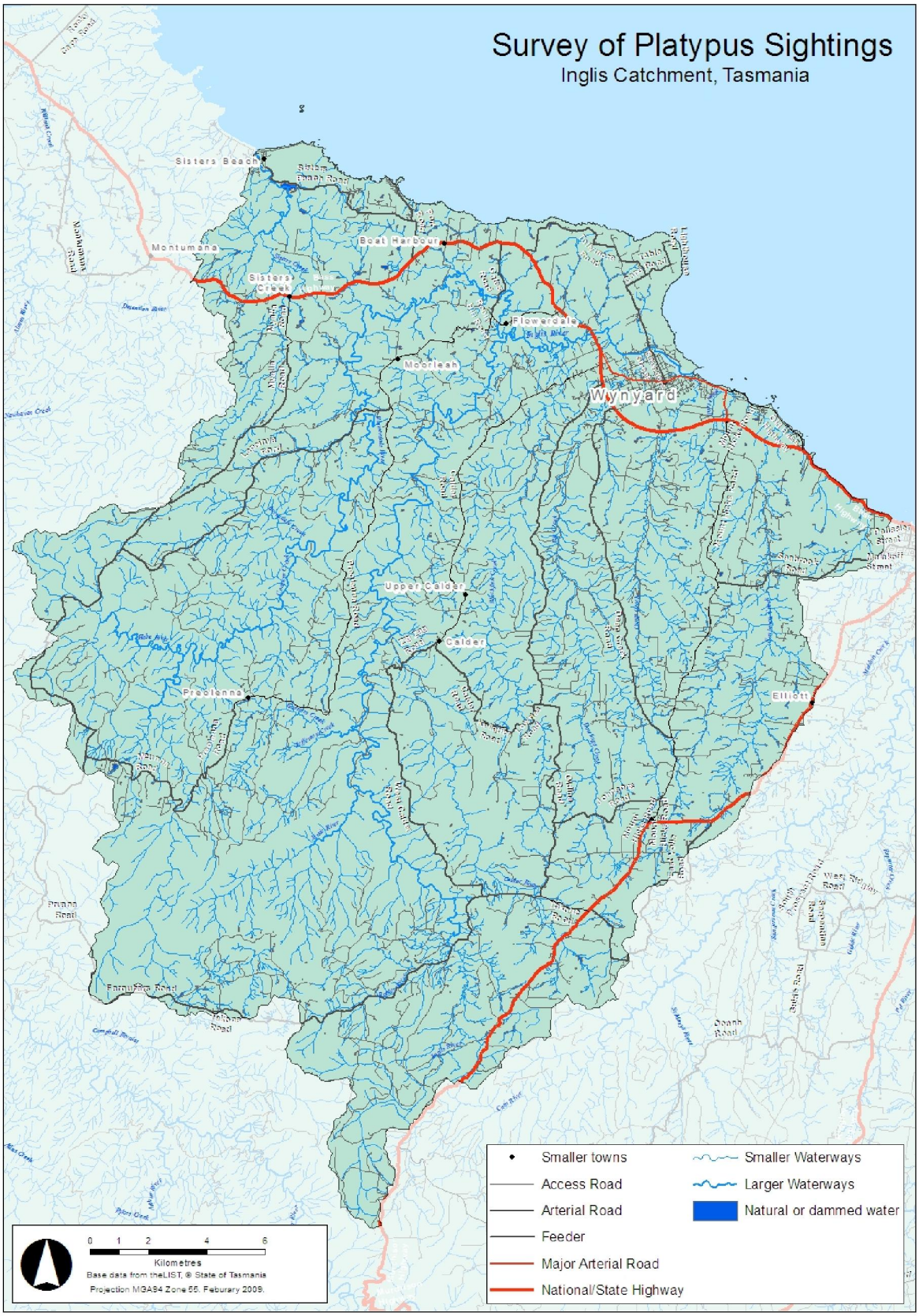
1. Waterbody type (eg River, Creek, Dam)	
2. Waterbody name	
3. Location of site	Grid ref (if known):
4. Have you seen a platypus at this site? (please tick)	1.1.2012 - 31.12.2012: o Yes o No Before 1.1.2012 : o Yes o No
5. If yes, what has been the frequency of these sightings? (please tick)	1.1.2012 - 31.12.2012: o Rare o Common o Don't know Before 1.1.2012 : o Rare o Common o Don't know
6. Approximate date and time of day of sightings (if known)	1.1.2012 - 31.12.2012: Before 1.1.2012 :

Site 1 (Please mark with a cross and a "1" on the map overleaf)

1. Waterbody type (eg River, Creek, Dam)	
2. Waterbody name	
3. Location of site	Grid ref (if known):
4. Have you seen a platypus at this site? (please tick)	1.1.2012 - 31.12.2012: o Yes o No Before 1.1.2012 : o Yes o No
5. If yes, what has been the frequency of these sightings? (please tick)	1.1.2012 - 31.12.2012: o Rare o Common o Don't know Before 1.1.2012 : o Rare o Common o Don't know
6. Approximate date and time of day of sightings (if known)	1.1.2012 - 31.12.2012: Before 1.1.2012 :

We would be grateful if you could provide a means of contact in case we would like further details on the information you have provided. This information
will only be used by James Macgregor and only to contact you in relation to this survey. You will not be identified in any reports or publications based on
the results of this survey and your details will not be passed on to any other individuals or organisations.
Your details:

Name:	Telephone: (H)
Address	(W)
	Email:


Survey of Platypus Sightings
Inglis Catchment, Tasmania
Sisters Beach
Montumana
Boat Harbour
Sisters Creek
Flowerdale
Moorleah
Wynyard
Upper Calder
Calder
Preolenna
Elliott
Smaller towns
Access Road
Arterial Road
Feeder
Major Arterial Road
National/State Highway
Smaller Waterways
Larger Waterways
Natural or dammed water
0 1 2 4 6
Kilometres
Base data from theLIST, © State of Tasmania
Projection MGA94 Zone 55, Feburary 2009.

Appendix E

Novel use of in-stream microchip readers to monitor wild platypuses

This Appendix has been published as an original research paper in Pacific Conservation Biology. Citation:

Macgregor J, Holyoake C, Munks S, Connolly J, Robertson I, Fleming P, Warren K (2015) Novel use of in-stream microchip readers to monitor wild platypuses. *Pacific Conservation Biology* 20(4): 376-384.

E.1 ABSTRACT

A variety of techniques have been used to monitor platypus populations to assess the impacts of the threats they face, but each technique has limitations. In this study we investigated the novel use of in-stream microchip readers, to remotely monitor the movements of microchipped wild platypuses. Over 13 months, we recorded movements of 18 microchipped individuals past nine fixed locations in the Inglis Catchment in northwest Tasmania, using three units of which all were capable of detecting Trovan® unique microchips and two were additionally capable of detecting ISO microchips. Each site was monitored one or two times, for durations of 8-39 days. We undertook direction of movement investigations during two monitoring periods, by placing the antennas from two systems in the same creek within 3 m of each other. In a total of 264 days of monitoring, 528 platypus observations were made from 18 individual platypuses, consisting of 13 of 18 (72%) platypuses captured at the monitoring sites within 16 months prior to monitoring, two platypuses captured at other sites in the same time period, and three of seven (43%) individuals microchipped 3-5 years previously. This number of platypus observations, in combination with the stable number of platypuses observed per day, the range of movement behaviours recorded and the results of the direction of movement investigations, indicates that at appropriate sites, in-stream microchip readers are an effective method of monitoring the movements and survivorship of microchipped wild platypuses.

E.2 INTRODUCTION

Platypuses are semi-aquatic mammals that are found in association with lakes, rivers and streams in Eastern Australia (Grant and Temple-Smith, 2003; Grant, 2009). Numerous observed and potential threats to platypus conservation have been reported, including habitat degradation, river flow alteration and disease (Grant and Temple-Smith, 2003; Gust and Griffiths, 2010; Serena and Williams, 2010), highlighting the importance of monitoring this cryptic species to assist with the development of conservation management plans. Platypuses have been monitored using live capture-release studies, radiotelemetry, data loggers and, to a lesser extent, remote observational studies, camera traps and acoustic transmitters (Serena, 1994; Bethge *et al.*, 2003; Grant, 2009; Gust and Griffiths, 2010; Griffiths *et al.*, 2013). At the time of writing the use of acoustic transmitters has not been reported in detail, however, each of the other listed methods of monitoring platypuses has limitations (Grant, 2009; Gust and Griffiths, 2010). For instance, while live capture studies provide detailed information on individuals, they are very labour intensive and often have low recapture rates - 36% of 271 males and 51% of 429 females over ~30 years in the Upper Shoalhaven River reported by Grant (2004), and 58% of males and 73% of females over ~12 years near Melbourne and 38% of males and 31% of females over 8 years in the Wimmera River reported by Serena and Williams (2013). Low recapture rates make it difficult to follow individuals through time and may in part be a result of net avoidance (Griffiths *et al.*, 2013). Similarly, while radiotelemetry and dataloggers provide detailed information on activity patterns, their use is limited by battery life, problems associated with application of the device and difficulties of recapture for device retrieval (Serena, 1994; Serena *et al.*, 1998; Bethge *et al.*, 2009). Devices are most commonly applied by glueing them to the fur which can cause skin irritation (S.Munks, unpublished data), and

although Bethge *et al.* (2001) found that data loggers did not significantly increase platypus foraging metabolic rate in a captive situation, potential adverse effects of attaching a device of up to 4.4% bodyweight to a platypus remain unknown (Bethge *et al.*, 2003). Camera traps can only be used to monitor animals when they move across land, not in water (Olssen-Herrin, 2009) and, like observational studies and public surveys, do not enable individuals to be identified.

The platypus is legally protected throughout its distribution (Gust and Griffiths, 2010) and is listed as Endangered in South Australia where it had a limited distribution at the time of European settlement (Grant, 2009). It is not listed under any other Australian state or federal threatened species legislation, is a species of "Least Concern" on the International Union for the Conservation of Nature and Natural Resources (IUCN) red list of threatened species and continues to have a similar distribution to that at the time of European settlement (Grant, 2009). However, because of the difficulties of monitoring platypuses described above, population declines are hard to identify and Grant (2009) suggested that the species could be more appropriately placed in the "Data Deficient" category by the IUCN.

In-stream antennas capable of remotely detecting implantable animal transponders (microchips) are commonly used to monitor individuals within wild fish populations (Zydlewski *et al.*, 2006). Similarly, antennas are used out of water to monitor other species of wildlife such as penguins and bats which either pass through or can be directed to pass through a small aperture (e.g. cave openings, fence apertures) (Kerry *et al.*, 1993; O'Donnell *et al.*, 2011).

Platypuses rest in burrows on land which they typically leave once a day to forage (Serena, 1994; Bethge *et al.,* 2009). While out of their burrows platypuses tend to display foraging behaviour, diving to the water body floor to find prey interspersed with time on the water surface (Gust and Handasyde, 1995). Gust and Handasyde (1995) and Bethge *et al.* (2003) found a mean foraging durations of ~10 hr/day and 11.5 hr/day, respectively. During foraging trips, platypuses have been observed to move distances of a few hundred metres to several kilometres along rivers and/or streams and have been known to move over land to avoid obstructions such as waterfalls, culverts or meanders in rivers (Serena, 1994; Gardner and Serena, 1995; Gust and Handasyde, 1995; Munday *et al.,* 1998; Mooney and Spencer, 2000). In this study, we investigate the novel use of in-stream microchip readers as a remote, long-term and relatively non-labour intensive method of monitoring microchipped wild platypuses as they move along waterways during foraging.

E.3 MATERIALS AND METHODS

A field study was performed between November 2011 and December 2012, using in-stream microchip reader units to monitor the movements of wild platypuses past nine specific sites (A-I) in the Inglis Catchment in northwest Tasmania (Figure E.1). Each micro-chip reader unit (Units 1-3) consisted of an antenna capable of detecting microchips connected to a decoder (Trovan® LID 650; Trovan Ltd., Microchips Australia Pty. Ltd., Keysborough, Victoria) that stored microchip numbers and the date/time they were detected for subsequent download. Unit 1 used a Trovan®ANT612 antenna, which is a 475 x 400 x 35 mm panel capable of detecting Trovan®Unique microchips passing within 250 mm of its flat surface. The antenna was placed on the floor of small waterways with the aim of detecting platypuses moving over the top of it

(Figure E.2a). Units 2 and 3 used Trovan®ANT C600 antennas, which are open-ended cylinders (used as a swim-through tunnel) 600 x 300 x 10 mm (diameter x depth x thickness). These antennas were placed with part of their circumference resting on the floor of small waterways, partly out of the water and with the water flowing through it, with the aim of detecting platypuses passing through the antenna (Figure E.2b). Unit 2 was configured to optimally detect Trovan®Unique microchips (but also capable of detecting ISO microchips) while Unit 3 was configured to optimally detect ISO microchips (but also capable of detecting Trovan®Unique microchips). At most sites, rocks and/or pieces of wood found nearby were placed around the antenna in an attempt to discourage platypuses from moving around it. Each unit was powered by a 12 Volt battery. In the early stages of the study, these batteries were changed and recharged daily; later the charge was maintained using a 135W Kyocera® Solar Panel and Plasmatronic® Dingo 20/20 Solar Regulator.

Figure E.1. Locations of platypus (*Ornithorhynchus anatinus*) capture and monitoring sites in the Inglis Catchment, Tasmania, a) red dots: animals identified with Trovan Unique® microchips (August 2011–December 2012), letters: sites monitored using in-stream antennae between November 2011 and December 2012; b) purple dots: animals identified with ISO microchips (December 2007–August 2008), letters: sites monitored using in-stream antennae capable of detecting ISO microchips between November 2011 and December 2012.

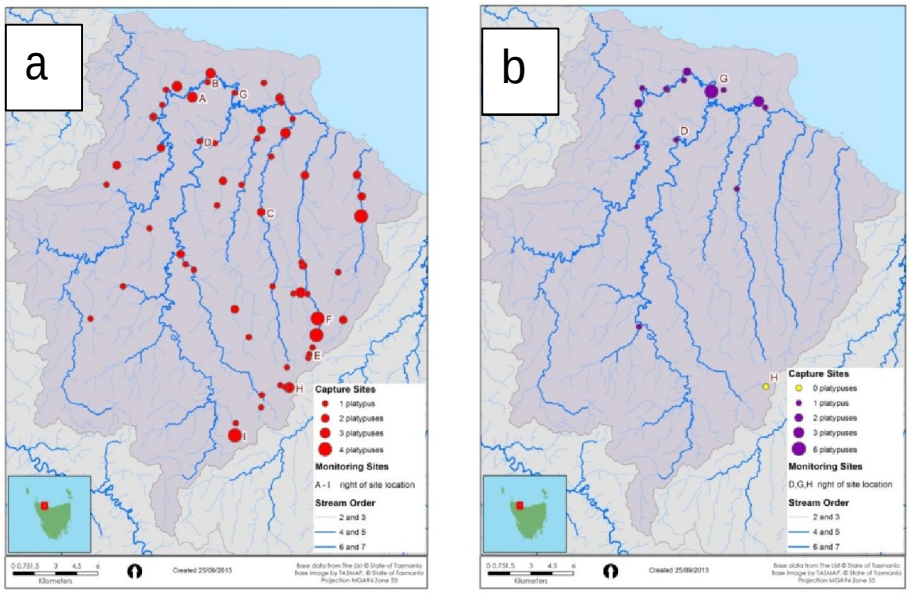
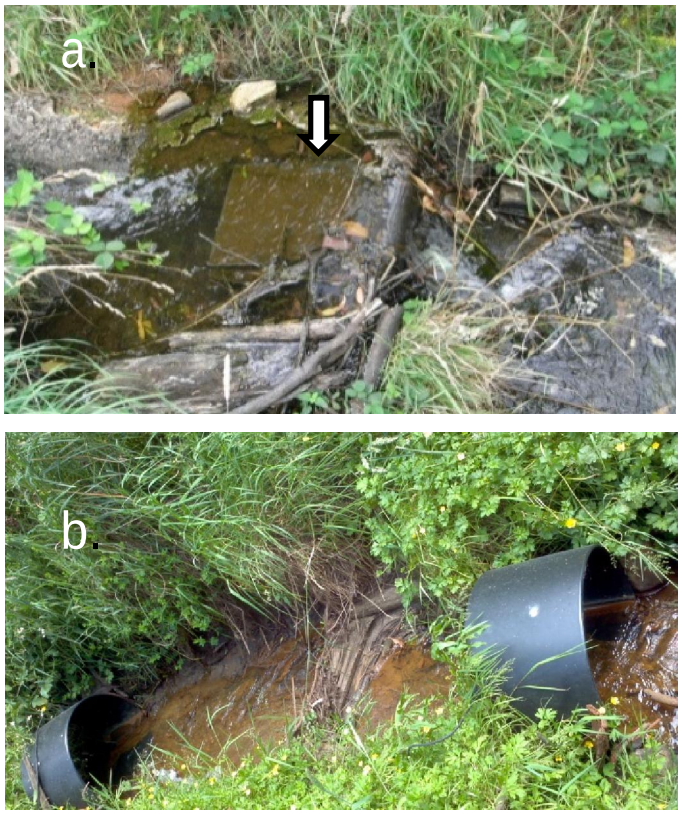


Figure E.2. Showing the three Units placed in the field. a) Unit 1, the ANT612 flat panel antenna (arrow) in the creek at Site A, and b) Units 2 and 3 (C600 swim-through tunnels) placed in line at Site G.



A total of 31 platypuses had been microchipped in the Inglis Catchment before commencement of the in-stream microchip monitoring: 23 (10 adult males, two juvenile males and 11 adult females) between December 2007 and August 2008 with ISO microchips (Macgregor *et al.*, 2010) and 8 (six adult males and two adult females) between August 2011 and November 2011 with Trovan Unique® microchips. During the period of in-stream monitoring, a further 80 platypuses (39 adult males, three juvenile males, 36 adult females and two juvenile females) were microchipped with Trovan Unique® microchips bringing the total number of animals microchipped in the study area to 111 by the end of this study (Figure E.1). Sites to locate the micro-chip readers were selected where at least one platypus had been captured and microchipped since August 2011 and where a section of the creek was a similar width to that of the

antennas and less than 25cm in depth. Each site was monitored for one or two periods of 8-31d duration; the exact length of each monitoring period depended on the logistics involved in transport of equipment, stock rotation through paddocks (where fieldwork sites were adjacent to pasture), and periods of flood.

Direction of movement investigations were performed in two 3-week monitoring periods (one at site D, one at site G) by placing the antennas from two monitoring units in the same creek within 3 m of each other (Figure E.2b). A recording of the same microchip from two units within 1 min of each other was considered to reflect movement of a platypus along the creek. Comparison of the time of recordings from the two units allowed us to determine the direction of movement of platypuses each time they were recorded. When only one of the two units recorded a microchip, examination of the direction of movement on previous and subsequent recordings allowed us to determine if the recording missed was due to the platypus turning around when it encountered the first antenna, or whether the passage of a platypus went undetected by one of the units.

The microchip reader units monitor constantly until a microchip is detected, after which monitoring is suspended for a pre-set wait time before continuous monitoring is recommenced. During the two first monitoring periods (which were at Site A), wait times of 0.1, 1 and 5 s were tested on different days. Subsequently, at the other sites, the wait time was set at 10 s.

Data from the microchip readers were used to determine two parameters. The first parameter was a ‘microchip recording’, which was defined as a single record of a

microchip where one unit was in place, or a record of the same microchip from the two units and within 1 min during the direction of movement investigation. In order to avoid over-representation of observations of any platypuses that might backtrack briefly for any reason as they move along a creek and be recorded more than once in a particular passage, a second parameter of "platypus observation" was used. Any two microchip recordings of the same platypus separated by <30 min (from a single unit or from two units in the same creek) were classed as a single platypus observation. The same principle was applied to any number of microchip recordings for the same platypus where consecutive intervals were <30 min. So when a platypus observation consisted of multiple microchip recordings, the total duration of the event may have been >30 min.

A day of monitoring was defined as an in-stream microchip reader unit monitoring one waterway for 24 h, or two units monitoring the same waterway within 3 m of each other for 24 h.

A type III mixed-model ANOVA test (with day of monitoring period and site as random factors) was carried out to test for an effect of time and monitoring site on the daily number of platypus observations. A second type III mixed-model ANOVA test (with day of monitoring period and platypus identity as random factors) tested for daily and individual platypus differences in activity patterns. Statistical analysis of results was performed using Statistica 8.0 (Stat Soft Inc. Tulsa OK, USA).

E.4 RESULTS

In a total of 264 days of monitoring, 528 platypus observations were made of 18 individual platypuses (9 males, 9 females) (Table E.1). Three of the seven platypuses

(43%) originally captured in 2007-8 and identified with ISO microchips, were detected at sites monitored in this study by units 2 and 3 (all at site G). Of the 18 platypuses captured since August 2012 at Sites A-I, and identified with a Trovan®Unique microchip, 13 (72%) were detected at the site of their capture. Two other platypuses microchipped in the associated health study were also detected: one at Site E ~200 m from the site of its capture in the same creek but separated by a small farm dam, and one at Site A, ~8 km by waterway from the site of its capture.

Table E.1. Numbers of microchipped platypuses in the study area and detected in this study.

	Adult male	Juvenile male	Adult female	Juvenile female	Total
Number of platypuses with ISO microchips implanted at monitored sites (2007-2008)	4	0	3	0	7
Number of platypuses with ISO microchips detected at the site of their capture in this study	3	0	0	0	3
Number of platypuses with Trovan® Unique microchips implanted at monitored sites (Aug 2011-Dec 2012)	8	0	10	0	18
Number of platypuses with Trovan® Unique microchips detected at the site of their capture in this study	5	0	8	0	13
Number of platypuses with Trovan® Unique microchips implanted away from monitoring sites (Aug 2011-Dec 2012)	37	3	28	2	70
Number of platypuses with Trovan® Unique microchips detected away from their capture site in this study	1	0	1	0	2

The mean number of times that individual platypuses were observed per day over the duration of each monitoring period is shown in Figure E.3 (the nine platypuses detected over two monitoring periods are each represented twice). In general, female platypuses

were observed more frequently than males. Figure E.4 shows examples of the patterns of observations that were recorded. Two platypuses showed a very regular pattern of observation timings, one female with a 24 h cycle and one male with a 48 h cycle; other individuals showed less regular patterns. Mixed-model ANOVA (with day of monitoring period and site as random factors) showed that the number of platypus observations varied between sites ($F_{8,225}$ = 34.07, p<0.001); there was a noticeably greater number of platypus observations at site I where four microchipped individuals were monitored. However, there was no effect of length of time that the sites were monitored ($F_{30,225}$ = 0.44, p>0.99). Mixed-model ANOVA (with day of monitoring period and platypus identity as random factors) showed that the number of platypus observations varied between individual platypuses ($F_{17,497}$ = 11.39, p<0.001), but importantly there was no effect of length of time that the sites were monitored ($F_{30,497}$ = 0.68, p=0.90).

Figure E.3. Mean number of platypus observations/day for each platypus organised from largest to smallest with positive standard deviation error bars.

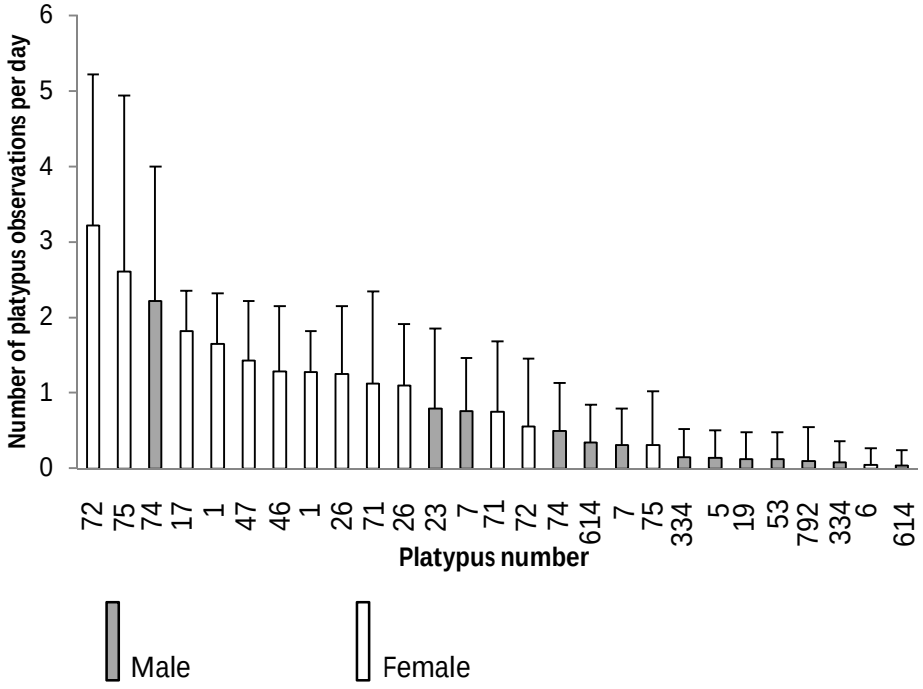


Figure E.4. Observations over 10 days at three sites (A-C) showing three individuals (Platypuses 7, 17 and 23) that were recorded regularly and two that were recorded only once or twice (Platypuses 5 and 6).

The incidence of multiple microchip recordings was reduced from 100% at the two shortest wait times (0.1 s and 1 s), to only 8% when the wait time was set at 10 s (Table E.2). For those multiple observations that occurred when the wait time was set at 10 s, both the swim-over panel and the swim-through tunnels recorded similar incidences (Table E.3).

Table E.2. Percentage of platypus observations that were classified as single or multiple microchiprecordings for different reader wait times.

Wait time	n observations	Single recordings	Multiple recordings	Duration of multiple microchip recordings*
0.1 s	17	0 %	100 %	2 – 7 s
1 s	1	0 %	100 %	2 – 7 s
5 s	7	57 %	43 %	5 – 33 s
10 s	503	92 %	8 %	10 s - 110 min

* Although the minimum interval between two platypus observation was set at 30 min, where consecutive intervals were <30 min, consecutive recordings were not considered independent and were classed as one platypus observation. The longest total duration of a single platypus observation was 110 min and consisted of ten sequential microchip recordings.

Table E.3. Percentage of platypus observations that were single or multiple microchip recordings for different antenna(s) when the wait time was set at 10 s.

Antenna(s) in creek	n observations	Single recordings	Multiple recordings
Flat panel only	400	91%	9%
Circular only	52	94%	6%

Data from eight days of one of the direction of movement investigations is shown in Figure E.5 to illustrate how the results have been interpreted. As shown in Table E.4, of 48 passages of a platypus in the direction of movement investigations, 41 (85%) were detected by both antennas and seven were only detected by one of the antennas (six by the flat panel antenna and one by the swim through antenna). On one occasion a platypus turned around after encountering the antenna (swim through antenna). The minimum time between two passings of a platypus in opposite directions during the two monitoring periods where direction of movement could be determined was 1 h 15 min 24 s.

Figure E.5. Direction of movement of platypus 26 over eight days at site D.

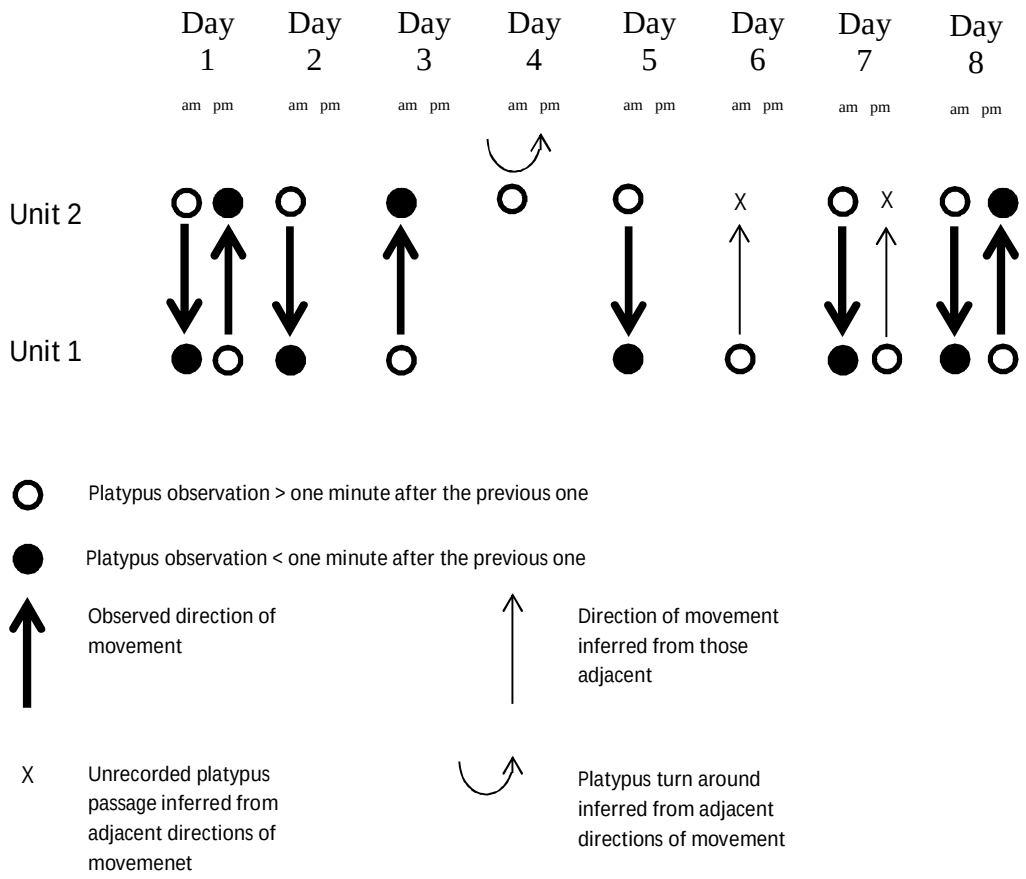


Table E.4. Number of times a platypus turned around when encountering an antenna and number of times an antenna failed to detect a platypus moving past it during direction of movement investigations.

		Number of observations		
		Unit 1	Unit 2	Unit 3
Site D	n obs	19	17*	
Platypus # 26 (20 d)	failed	1[+]	5	
	Turned around	0	1	
Site G	n obs		28	27
Platypus #1 (22 d)	failed		0	1
	Turned around		0	0
	% failures	5%	10%	3.6%
	% turn arounds	0%	2%	0%

*Excluding the first recording by Unit 2 at site D which could not be characterised.

[+]Excludes one occasion when the batteries were changed late and the one supplying unit 1 (which drew more power than unit 2) had run out of power.

E.5 DISCUSSION

Results of this study indicate that in-stream microchip readers are an effective method of detecting microchipped wild platypuses at appropriate sites. Importantly, during the 13 months of the study, the detection rates of platypuses microchipped at the monitoring sites (72% of the platypuses microchipped in 2011-2012 and 43% of those microchipped in 2007-2008) were similar to the recapture rates achieved during repeated live capture studies performed by Grant (2004) over ~30 years and Serena and Williams (2013) in two areas over ~8 and 12 years. The results of the direction of movement investigations, the absence of a significant effect of length of monitoring on the number of platypus observations at each site and for each individual platypus, and the regular and frequent observations from two platypuses further reinforce our conclusion.

Suggested causes of failure to recapture certain individuals during longitudinal live capture studies have focussed on a likely high degree of mobility of certain individuals, including individuals with large ranges, individuals with a nomadic or roving breeding strategy, non-breeding individuals unable to find a vacant home range, and transient occupation of an area (Grant, 2004; Bethge *et al.*, 2009; Serena and Williams, 2013). Such explanations would be consistent with certain platypuses not being detected in this study. The range of frequency and regularity of observations from the 18 platypuses that were detected is also consistent with the findings of previous studies. Firstly, a range of behaviour patterns have been observed using radiotracking and dataloggers - some very regular, others less so (Gardner and Serena, 1995; Bethge *et al.*, 2009). Secondly, radiotracking has shown platypuses using certain parts of their home ranges more frequently than others (Gardner and Serena, 1995; Gust and Handasyde, 1995). Lastly, a

long-term mark-recapture study found that the home ranges of male platypuses were significantly larger than those of females (Serena and Williams, 2013). The variation of frequency of observations for those individuals that were detected in this study (Figure E.3) is likely to be a result of the differing home range sizes of the individuals (affected in particular by their sex) and the position of the antenna within each platypus's home range. It should be noted that we did not attempt to determine whether platypuses ever left the water to avoid the antennas and this remains a possible explanation, at least in part, for the failure to detect certain platypuses and for the variability in detection frequency in those that were detected.

The observation at Site G of three platypuses microchipped in 2008 is of particular importance. This observation reveals that these individuals were still present at the sites, despite not being re-trapped during the associated health study (four nights of trapping at that site between August 2011 to December 2012; Macgregor *et al.*, unpublished data). Without the use of the in-stream antennas, the continued presence of these animals would not have been known.

The direction of movement investigations suggested that microchipped platypuses were recorded on 93% of occasions that they passed an antenna. Of the remaining 7% of passages, it was not possible to determine if the absence of a recording was due to the equipment failing to detect a microchip that passed within its read range or due to platypuses leaving the water to move around the antennas. While comparison of Unit 1 with Units 2 and 3 may indicate that the flat panel (pass-over) antennas are more efficient than the circular (pass-through) antennas, the differences in efficacy of the two antennae designs is not great. Furthermore, the ability of the pass-through antennas to

detect both ISO and Trovan Unique microchips will be important at many survey locations.

We observed signs that on some occasions the antennas appear to alter platypus behaviour. Firstly, in the six weeks of our direction of movement investigations we identified one platypus turning around after encountering an antenna. Secondly, we observed multiple microchip recordings over periods longer than would be expected for a platypus moving through the read range of the antennas. Such multiple microchip recordings may have been produced by a platypus moving very slowly past an antenna or moving up and down a short section of creek during foraging. However, it may also indicate that some platypuses spent time investigating the antennas, since sight or touch may alert platypuses to presence of the antennas. Platypus 23 appeared to investigate the antenna at Site C, despite this antenna being covered in river substrate, suggesting that platypuses may sense the electric field produced by the antenna using electroreceptors in their bill that are usually used to detect prey (Schleich, 1986).

A read-wait time of 10 s after a microchip was detected was settled on for this study, to reduce time platypuses might be aware of the electric field and reduce the number of multiple microchip readings evident when shorter wait times were tested. The time taken for a platypus to pass through the field of the antenna when moving at normal speed along a creek is likely to always be greater than 0.1 and 1 s and may even be longer than 5 s. However, it is unlikely that a platypus moving normally should take longer than 10 s to pass over/through an antenna.

We considered consecutive microchip recordings separated by <30 min as not independent to ensure that we did not overanalyse our data. The choice of any particular interval could be debated but we chose 30min as a likely maximum time that a platypus would spend either foraging in a section of a narrow creek or investigating the antenna. The use of a figure close to this is supported by the following points: 1) clusters of three or more microchip readings separated by up to several minutes were observed on several occasions, indicating that the platypuses were not simply moving in a straight line up the creek and were sometimes returning and passing back over/through antennae; and 2) the shortest interval between return journeys during apparently normal behaviour during the direction of movement investigations was 1hr 15min 24s. However, it is likely that whatever time delay is chosen, occasionally two platypus observations will be miscounted as one, or a single platypus observation will be miscounted as more than one.

The use of in-stream microchip readers does not overcome all of the obstacles facing platypus monitoring. Importantly it is only applicable in relatively small creeks; although it may be that experimentation with antenna design may allow this method to work in wider and deeper creeks. Other limitations of in-stream microchip readers are that they only provide information about platypus movements at certain locations, they provide no information on the observed individuals' health except that they are alive, and a live capture and release study is required to microchip individuals before the units can be used. Also, while the equipment is robust it is possible that the antennas could be moved or even damaged by fast flowing water if not secured adequately and there is potential for the electronics in the decoders to be damaged by waterlogging if placed in a position where floodwater may reach. However, as a platypus monitoring technique,

this method comprises a unique set of advantages: it is reliable, remote and relatively non-labour intensive; requires only the routine implantation of a microchip; and can be used repeatedly or left in the field to monitor the same animal over periods of years. We believe that in-stream microchip readers will allow important new data to be gathered in many areas of platypus conservation research and assist in more reliably categorising the species according to threatened species schedules. Because platypuses have routinely been identified in research project with microchips for over two decades, it will be possible to use the technique to study platypuses captured in previous research projects, which may not have anticipated ongoing monitoring, as well as those in prospective studies. Specifically, we think in-stream antennas will assist in gathering information on platypus short- and long-term habitat use and home ranges, population demographics, survivorship and longevity, as well as the safety of new research techniques and net avoidance during live capture studies. We also think that survivorship and movement monitoring will aid the impact assessment of disease, including mucormycosis, and human land use practices.

APPENDIX F.

Preliminary investigation into platypus survivorship and longevity at Lake Lea, Tasmania.

F.1 INTRODUCTION

As described in Section 3.1, research into the survivorship and longevity of wild platypuses, has largely relied on recapture of individuals. The capture of two females with known ages of 21 and 13 years have been reported after initial capture as juveniles (Grant, 2004) and survivorships of up to 127 months (Serena and Williams, 2013) and 15 years (Grant, 2004) have been reported in individuals initially captured as adults.

Macgregor *et al.* (2015) (Appendix E) and Chapter 3 have described the use of in-stream microchip readers to detect platypuses microchipped up to six years previously in small creeks and have suggested that the method could be used to investigate platypus longevity and survivorship over longer periods of time. The first reported use of microchips to identify an individual platypus was on 19/12/1987 (Grant and Whittington, 1991). Since then, microchips have become the standard method of identification.

The aim of this preliminary study was to use in-stream microchip readers at a location where platypuses had been identified using microchips during a variety of studies carried out over a six year period more than a decade previously to determine whether any such individuals could be detected.

F.2 METHODS

Between 25/4/2013 and 26/5/2013, the antennas of two Trovan®ANT612/LID650 microchip reader units were placed in Bonds Creek (Otley, 1996) at the south western end of Lake Lea in northwest Tasmania 55409037E 5402058N (Figures F.1 and F.2), using methods described by Macgregor *et al.* (2015) (Appendix E).

Figure F.1. Lake Lea, Tasmania, and position of in-stream antennas used to remotely monitor the movements of platypuses (*Ornithorhynchus anatinus*). (source http://maps.thelist.tas.gov.au)

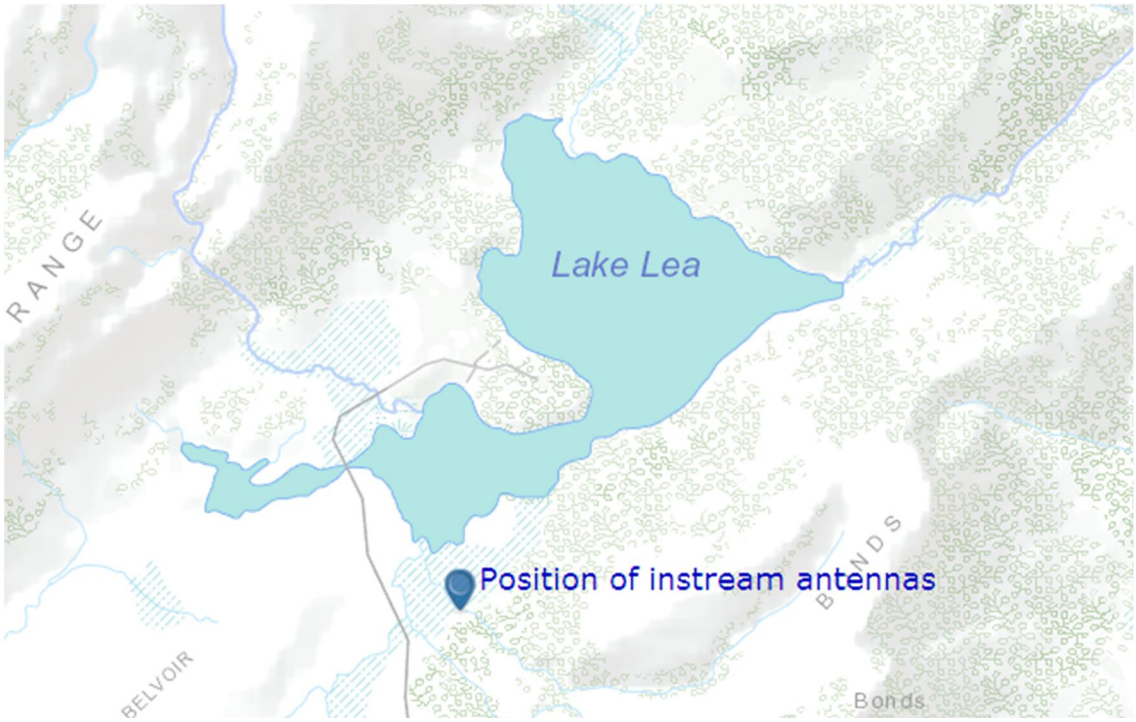
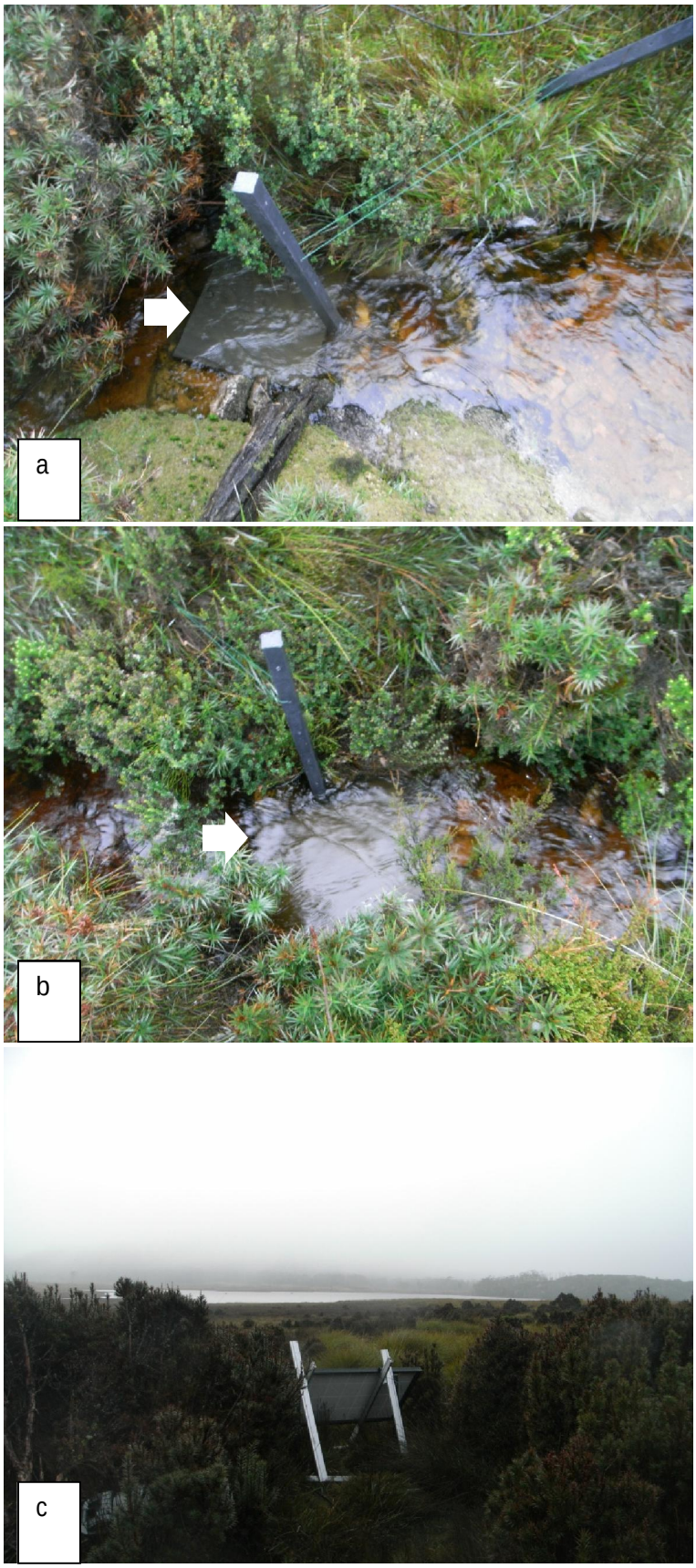


Figure F.2. a and b) position of antennas in creek - arrow, and c) Looking north from antenna postion, past solar panel and data logger box, to Lake Lea.



The potential study population consisted of 93 platypuses captured at various locations in and around the lake - 91 individuals first captured between 26/3/1996 and 3/2/2002 (Otley *et al.*, 2000; Bethge, 2002; Munks *et al*., unpublished data) and two first captured on 23/4/13 (Macgregor *et al*., unpublished data). Of these, 17 had been captured at least once within 400m of the monitoring site (S. Munks, unpublished data). Survivorship was calculated to the nearest month as the time that had elapsed between first capture of an individual and its last observation with the in-stream antennas. Age was calculated to the nearest year for each platypus detected by the in-stream antennas that had been a juvenile or subadult at the time of its first capture.

F.3 RESULTS

Three platypuses were detected during this monitoring period and their details are given in Table F.1.

Table F.1. Details of platypuses detected by in-stream microchip readers at Lake Lea.

Platypus	Date of first catpure	Age at first capture	Survivorship to last remote observation	Age during remote monitoring
AC	15.2.1998	Subadult	15 years 3 months	16 years
F0	28.2.1999	Adult	14 years 3 months	Unknown
D1	22.11.1998	Adult	14 years 6 months	Unknown

F.4 DISCUSSION

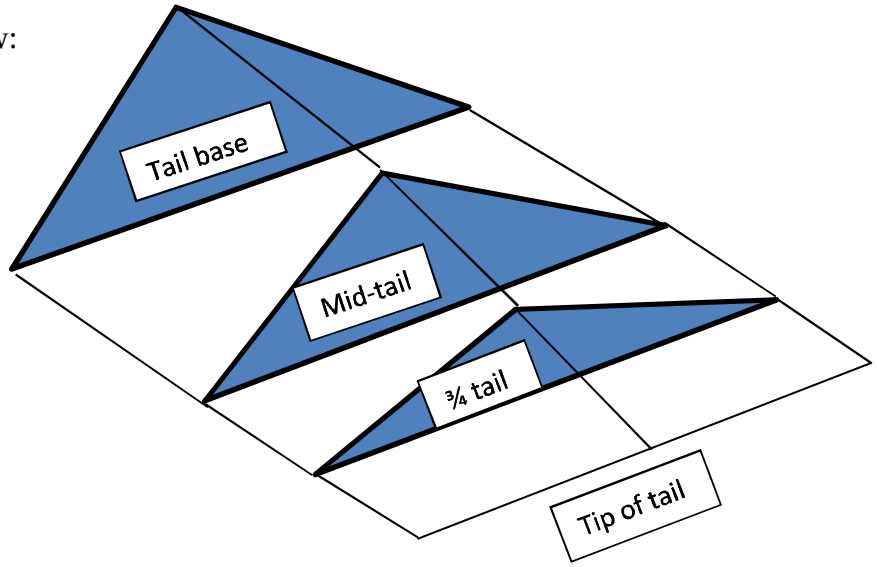
Platypus AC is the second oldest platypus of known age to have been reported (Grant, 2004). Platypuses FO and D1 are at the upper end of the range of survivorships reported for platypuses of unknown age (Grant, 2004; Serena and Williams, 2013). These results, and the fact that they were achieved with two days of fieldwork and without the need for live captures, further emphasise the research potential and welfare benefits of the use of in-stream microchip readers in the study of platypus ecology. Further research using

in-stream antennas at Lake Lea and at other sites were platypuses have historically been microchipped should provide additional insights into platypus longevity and survivorship.

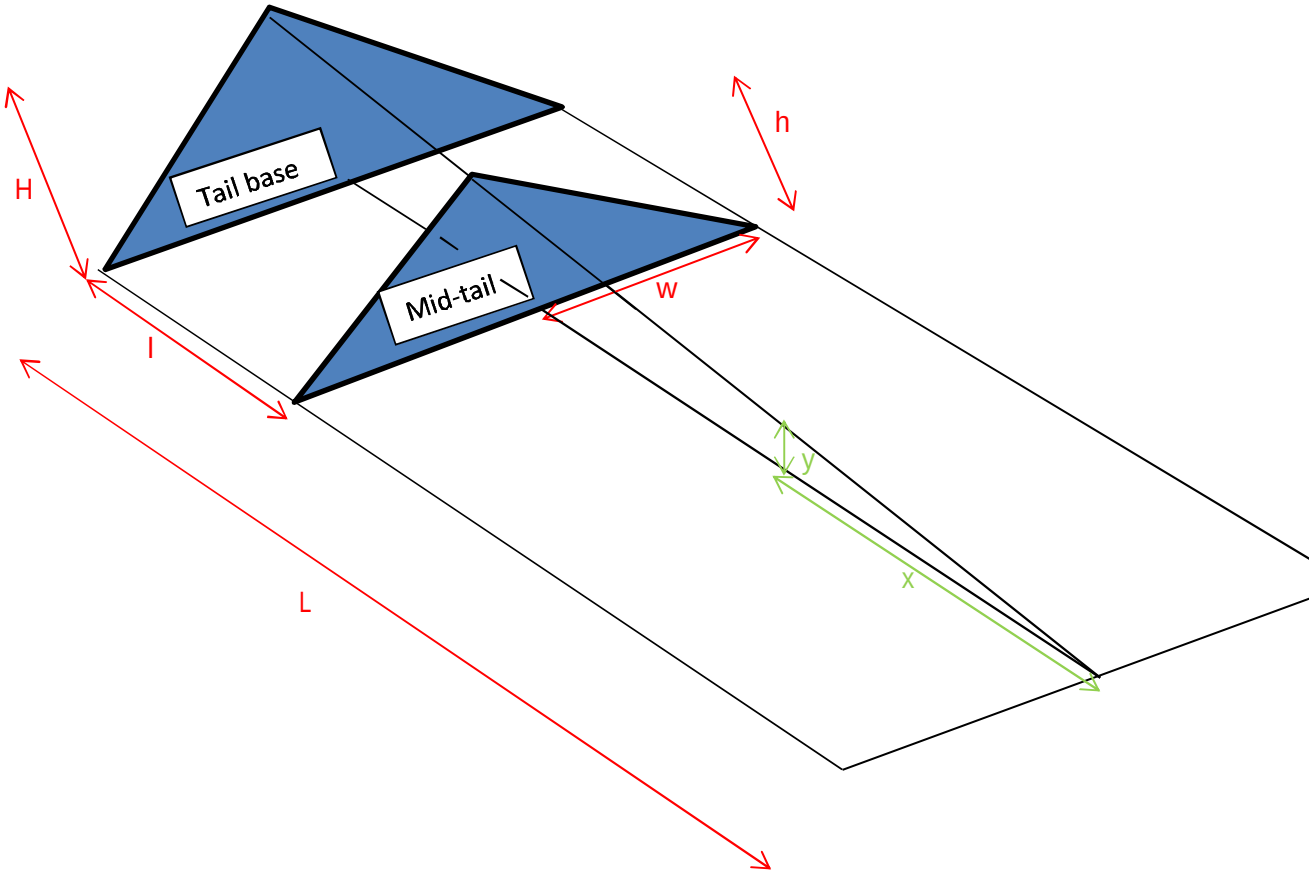
APPENDIX G.

Derivation of formula for calculation of tail fat volume using ultrasound measurements

To derive a formula for tail fat volume, the tail fat was approximated to three volumes (tail base to mid-tail, mid-tail to ¾ tail, and ¾ tail to tip of tail. Each of these was considered to be of constant width and to be triangular in cross section with a height that decreased at a constant rate. The rate of change of cross sectional height was not assumed to be the same between the three volumes. The final cross sectional height, at the tip of the tail was assumed to be 0. The approximation to the tail shape is shown below:



To derive a formula for the tail fat volume a formula for the three component volumes was determined. To do this, only one volume needs to be considered as below:



The area of the triangle at the tail base = Hw **Equation 1**

The area of the triangle at the mid-tail = hw **Equation 2**

l = 2 * quarter tail length **Equation 3**

The gradient of the line along the apex of the triangular volume is: (H-h)/l **Equation 4**

At any point along the shape, the relationship between the height of the shape at the apex (x)and the distance from the tip of the shape (y) is: y = (H-h)x/l **Equation 5**

Hence H = (H-h)L/l ⇨ L = Hl/(H-h) **Equation 6**

At any point along the shape, the cross sectional area is: y*w

Substituting for y from Equation 5, cross sectional area = ((H-h)xw/l)

The volume of a small length (δx)of the shape is at a distance x from the tip is: ((H-h)xw/l) δx

The volume of part of the shape between any points a and b along its length is:

$\Sigma((H-h)xw/L)\ \delta x$ for all the small volumes of width (δx) between a and b.

As δx approaches 0, the volume approaches $\int_a^b ((H-h)xw/l)\ dx$

In the case of the volume between the tail base and the mid-tail,

$[(w/2l)(H-h)x^2]_a^b$

$=(w/2l)(\ H-h)(L^2-(L-l)^2)$

$= (w/2l)(H-h)(\ L^2-\ L^2+2Ll\ -l^2)$

$=(w/2l)(H-h)(2Ll\ -l^2)$ (substituting for L from Equation 6)

$=(w/2l)(H-h)(2(Hl^2/(H-h))-l^2)$

$=(w/2l)(H-h)(2Hl^2-Hl2+hl2)/(H-h)$

$=(w/2l)\ (2Hl^2-Hl2+hl2)$

$=(wl/2)(H+h)$ (substituting from Equations 1 - 3)

= (Area of triangle at tail base + area of triangle at mid-tail)*(quarter tail length)

The same principle applies to the volumes other sections of the tail which are approximated as follows:

Mid-tail to ¾ tail = (Area of triangle at mid-tail + area of triangle at ¾ tail)*(quarter tail length/2)

¾ tail to tip of tail = (Area of triangle ¾ tail)*(quarter tail length/2)

Hence the overall tail volume is approximated to:

(Quarter tail length/2)*(2*(Area of triangle at tail base + area of triangle at mid-tail))+ (Area of triangle at mid-tail + area of triangle at ¾ tail)+ (Area of triangle ¾ tail)

= Quarter tail length/2*(2*Area of triangle at tail base) + 3*(area of triangle at mid-tail) + (2*area of triangle at ¾ tail)

= ((2* tail base fat area) + (3 * tail fat area) + (2 * ¾ tail fat area))*(tail length/8)
Equation 7

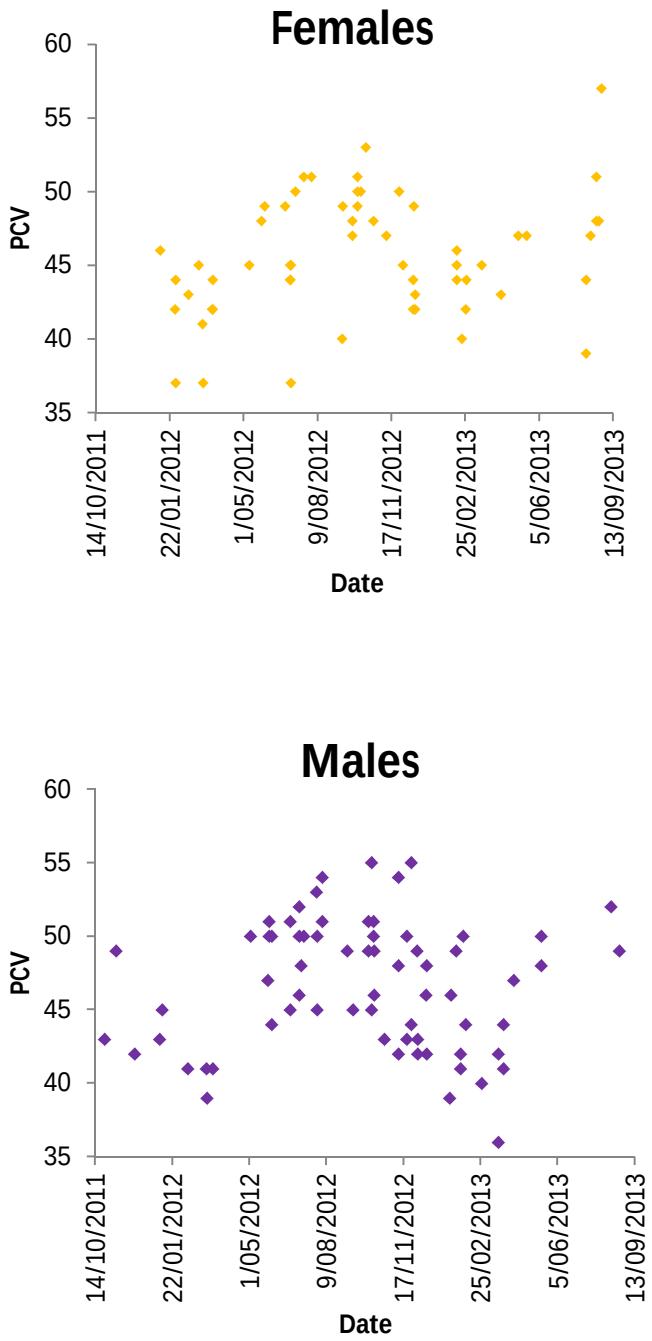
It is the use of Equation 7 that is described in Section 6.3.6.

Appendix H.

Reference curve development for a seasonally varying parameter

The steps below further illustrate the process, shown as part of Figure 7.1, of determining the nature of the reference curve for a seasonally varying parameter (PCV in females).

Step 2



Step S1 and S2

Highest R^2 value achieved with regression against sine wave with a period of one year and a minimum on 12th March, as follows:

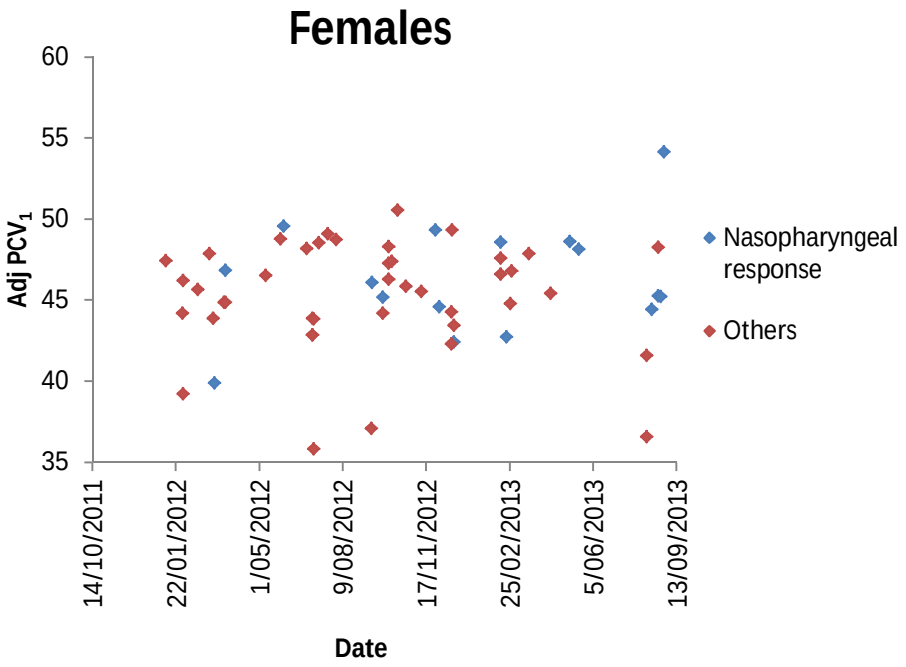
R= .54458535 R^2= .29657320 Adjusted R^2= .28401201 F(1,56)=23.610 p<.00001
Std.Error of estimate: 3.4469

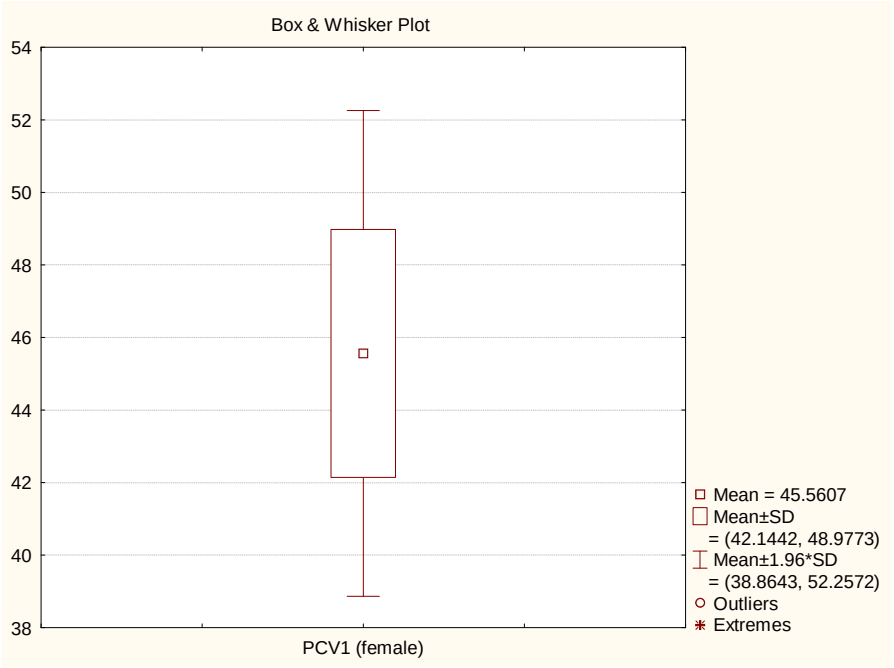
N=58	Beta	Std.Err. of Beta	B	Std.Err. of B	t(56)	p-level
Intercept			45.56073	0.452690	100.6443	0.000000
NewVar37	0.544585	0.112077	2.90539	0.597935	4.8590	0.000010

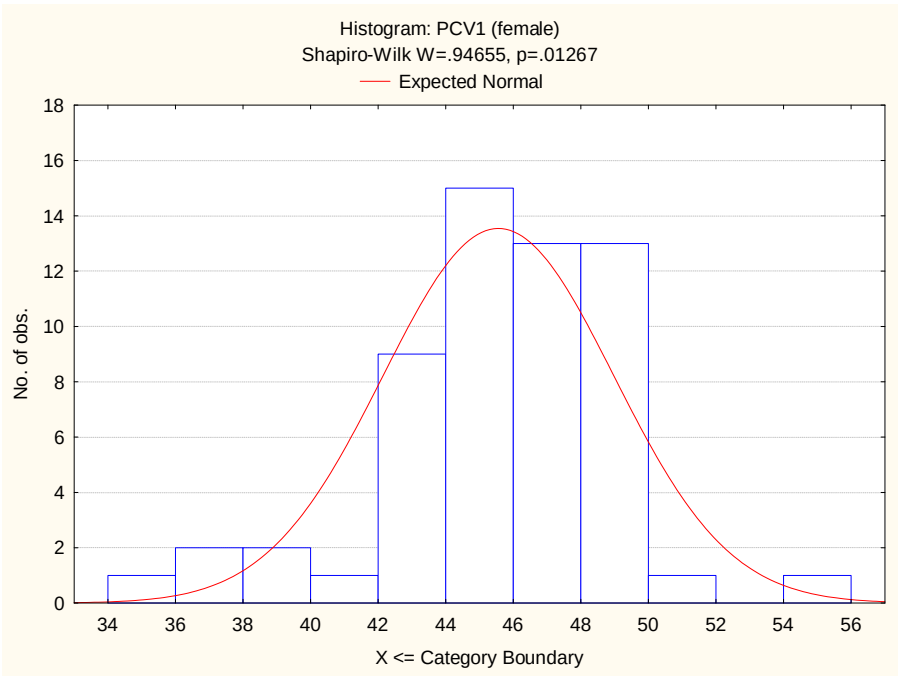
Step S3

PCV values adjusted according to the following equation

Adjusted PCV_1 = Observed PCV - (2.905 sine ((DOY - 203)*(360/365))







In both male and female platypuses, an individual that displayed a nasopharyngeal response under anaesthesia had the highest values for adjusted PCV_1, RCC_1 and Hb_1. Despite these results not being identified as outliers by Microsoft Excel, they were excluded as outliers (Step S4) because of the possibility that splenic contraction occurred in these individuals (see Section 7.4).

Step S1 and S2 (second iteration).

Highest R^2 value achieved with regression against sine wave with a period of one year and a minimum on 12th March, as follows:

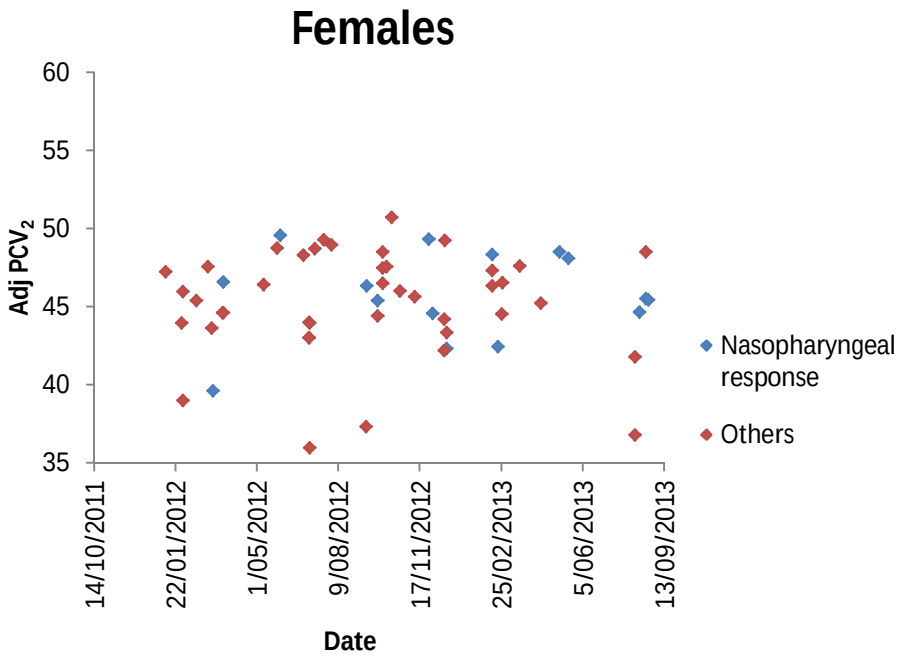
R= .52700152 R²= .27773060 Adjusted R²= .26459843 F(1,55)=21.149 p<.00003
Std.Error of estimate: 3.2689

	Beta	Std.Err. of Beta	B	Std.Err. of B	t(56)	p-level
N=57						
Intercept			45.40853	0.432982	104.8740	0.000000
NewVar37	0.527002	0.114596	2.65157	0.576581	4.5988	0.000025

Step S3 (second iteration).

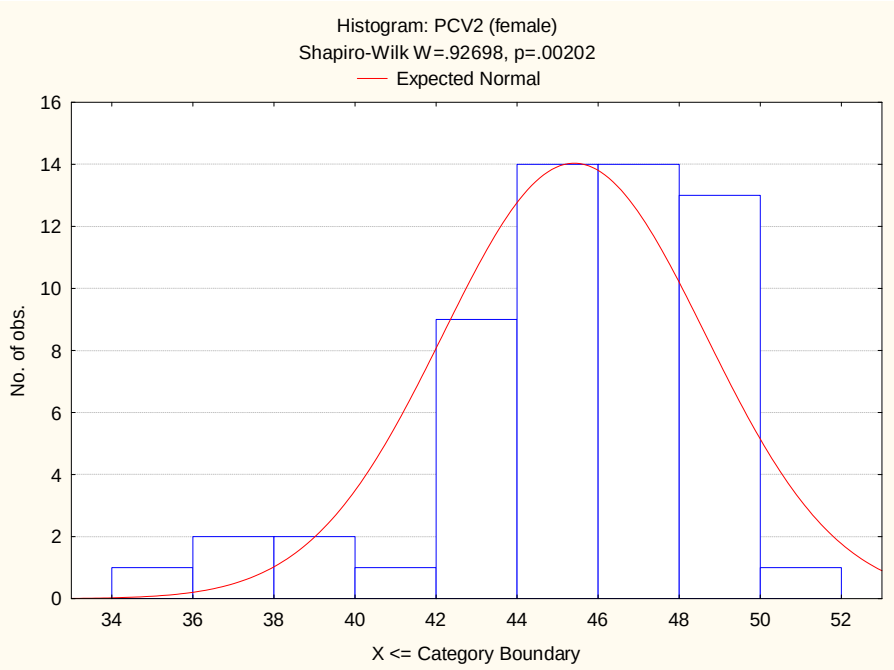
PCV values adjusted according to the following equation

Adjusted PCV_2 = Observed PCV - (2.652 sine ((DOY - 202)*(360/365))



No outliers identified above or on Box and Whisker plot/frequency histogram.

Step 7c



Data not normally distributed.

Step 8c

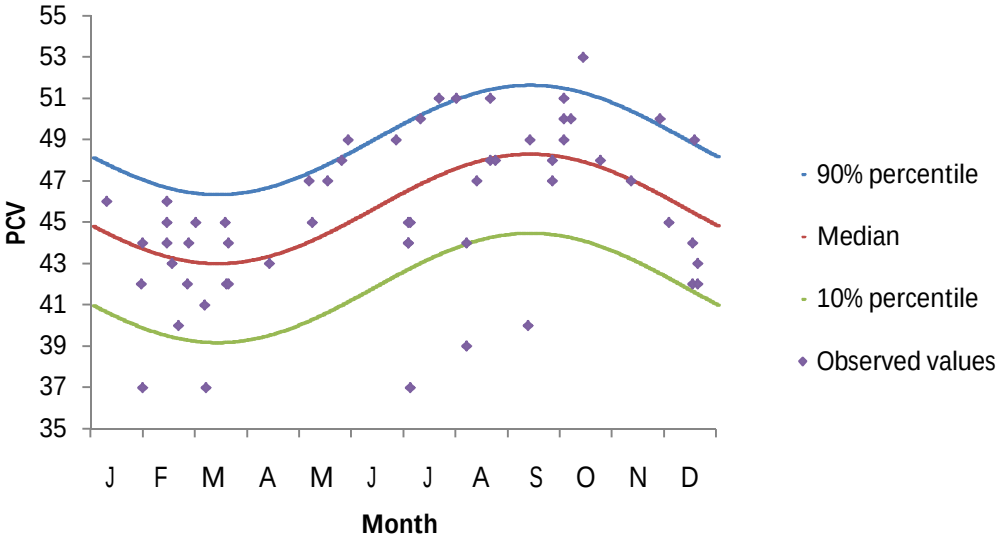
Distribution properties as follows:

	Valid N	Median	Minimum	Maximum	10 Percentile	90 Percentile
Adj PCV F	57	45.65	35.98	50.75	41.82	48.99

Distribution of PCV reported as, and seasonally varying reference range represented graphically in Figure 7.9 using, the following equation:

Reference mean/percentile = adjusted mean/percentile + (2.7*sine (DOY+202*(360/365)).

Females


55
53
51
49
47
45
43
41
39
37
35
PCV
J F M A M J J A S O N D
Month
90% percentile
Median
10% percentile
Observed values

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